VARIATIONS IN PROTEIN PROCESSING LEAD TO VARIATIONS IN STRUCTURE AND FUNCTION OF THE HIV-1 ENVELOPE PROTEIN

by

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Abstract

Mucosal transmission of HIV is a complex, inefficient event that leads to the seeding, and eventual disruption, of the gut-associated lymphoid tissue. The basis for the gut-tropic nature of acute HIV infection is unknown. The HIV envelope protein (Env) of early-replicating viruses encode several distinct transmission signatures believed to impart increased transmission fitness on these isolates. One such signature involves a reduced number of potential N-linked glycosylation sites underscoring the importance of glycosylation on the fitness of early-replicating isolates. Our group has previously shown that Env glycosylation plays a role in the interaction between the V2 domain of Env gp120 (V2) and the gut homing receptor, integrin $\alpha_4\beta_7$, expressed on HIV target $CD4^+$ T-cells. The $\alpha_4\beta_7$ binding epitope on the V2 overlaps with the RV144 Thailand HIV vaccine trial sieve residues associated with vaccine-elicited immune pressure. In this thesis, we present data showing that a second signature of transmission located in the signal peptide (SP) of Env can modulate the antigenicity of gp120 and influence viral neutralization sensitivity through altered glycan processing. Additionally, we show that deglycosylation of gp120 can increase the functional capacity of $\alpha_4\beta_7$ adhesion to the V2. We also show that mAbs from vaccination and natural infection targeting an alternate, helical conformation of the V2 that is absent from the SOSIP stabilized trimer inhibit the adhesion of $\alpha_4\beta_7$ expressing cells to V2 peptides. This conformation of the V2 is present on incompletely-processed Env that is expressed on the surface of infected cells and is incorporated into virions. This data suggests that incompletely-processed Env presents the V2 in an unconstrained, helical conformation that is capable of binding $\alpha_4\beta_7$. Taken together, the data presented in this thesis suggests a mechanism by which the virus can modulate glycosylation of the Env

protein without changing the primary amino acid sequence of the functional protein, and that relevant, incompletely-processed Env exposes the $\alpha_4\beta_7$ binding site on V2. This data supports the connection between Env glycosylation, the gut homing integrin $\alpha_4\beta_7$ and the transmission and pathogenesis of HIV. We believe this data is useful for HIV vaccine and therapy design.

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Committee Chair: Dr. Trina Schroer, Ph.D.

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List of Abbreviations

Ab	Antibody
ART	Antiretroviral Therapy
bnAb	Broadly Neutralizing Monoclonal Antibody
CD4i	CD4-Induced Conformation
Env	HIV-1 Envelope Protein
ER	Endoplasmic Reticulum
Fuc	Fucose
Gal	Galactose
GALT	Gut Associated Lymphoid Tissue
Glc	Glucose
GlcNAc	N-acetyl glucosamine
GNA	*Galanthas nivalis* lectin
HIV	Human Immunodeficiency Virus
M.W.	Molecular Weight
mAb	Monoclonal Antibody
Man	Mannose
MLN	Mesenteric Lymph Node
PBMC	Peripheral Blood Mononuclear Cell
PNGS	Potential N-Linked Glycosylation Site
PP	Peyer's Patch
PTM	Post-Translational Modification
PTP	Post-Translational Processing

RA	Retinoic Acid
RU	Response Units
SEC	Size Exclusion Chromatography
SP	Signal Peptide
SRP	Signal Recognition Particle
SRPr	Signal Recognition Particle Receptor

Chapter 1:
INTRODUCTION

1.1 Historical Context of the HIV Epidemic

In 1981, physicians in Los Angeles and New York began reporting clusters of cases presenting with previously rare lung infections (Pneumocystis carinii pneumonia) and unusually aggressive cancer (Kaposi's Sarcoma) in predominantly young men who have sex with men (MSM) [1, 2]. Survival of less than 20 months was reported for these patients, rather than the expected 8-13 years with such diagnoses. By the end of the year, a total of 270 cases of severe immune deficiency had been reported in MSM, 121 of which had died [3]. In 1982, the US Centers for Disease Control used the term AIDS, acquired immune deficiency syndrome, for the first time to describe this disorder of cellular immunity. In 1983, Francoise Barre-Sinoussi and Luc Montagnier at the Pasteur Institute published a report citing the isolation of a T-lymphotropic retrovirus and its potential as the causative agent for AIDS [4]. The following year, four articles all published in the same issue of *Science* by Robert Gallo and colleagues reported extensively on 1) the detection, isolation and production of what they designated HTLV-III from symptomatic AIDS patients, 2) the ability to frequently detect and isolate HTLV-III from AIDS and pre-AIDS patients, 3) the characterization of HTLV-III as a distinct relative of the other HTLV family members, and 4) the presence of HTLV-III-reactive antibodies in the serum of AIDS and pre-AIDS patients [5-8]. In 1986, HTLV-III was officially renamed HIV (human immunodeficiency virus). That same year, cases of HIV/AIDS had reached every major region of the world with a total of over 38,000 cases worldwide.

It wasn't until 1990 that the US FDA approved the first drug, zidovudine (AZT), to treat children with AIDS. By then, the infection had exploded globally to an estimated 8-10 million people living with HIV and over 300,000 documented cases of AIDS [9]. The

FDA approved Combivir in 1997, the first combination antiretroviral therapy (ART) to treat HIV infection. It wasn't until 2011 that the FDA approved the second all-in-one combination therapy option for people living with HIV. In 2012 the FDA approved PrEP, pre-exposure prophylaxis, for HIV-negative, at-risk individuals to prevent sexual transmission of HIV with the aim of reducing transmission by providing anti-retroviral therapy as a prevention therapy. With the aim of reducing transmission further, the WHO launched new treatment guidelines in 2015 recommending that all people living with HIV should receive antiretroviral therapy as soon as possible after their diagnosis, regardless of their $CD4^+$ T-cell count. Previously, people living with HIV were not put on therapy until they experienced a drop in $CD4^+$ T-cell count, leaving the chance that a viremic individual could transmit the virus to a partner. Subsequent studies of HIV discordant couples (HPTN 052 and PARTNER) showed that HIV transmission from people living with HIV that are virally suppressed by ART to undetectable levels is virtually zero [10, 11]. This finding has led to the adoption of the educational and anti-stigma slogan "U=U" (undetectable = untransmittable), meaning that a person living with HIV who is virally suppressed by ART to an undetectable viral load does not transmit the virus to their partners.

Even with the development of combination ART and transmission prevention strategies, the epidemic continues. In 2017, there was an estimated 36.7 million people living with HIV/AIDS worldwide, including 2.1 million children less than 15 years old. Every year an estimated 1.8 million individuals become newly infected – about 5,000 new infections every day. Unfortunately, only an estimated 70% of people living with HIV know their status, highlighting a gap in access to testing services. Additionally, only 20.9 million out of the estimated 36.7 million people living with HIV (~57%) are accessing

ART. Gaps in access to testing and treatment highlight the importance of developing an effective, robustly protective HIV vaccine.

Since the onset of the HIV epidemic there have been over 220 Phase I, II and III HIV vaccine clinical trials, a majority of which (140 trials) were conducted in the US [12]. Only 6 clinical HIV vaccine trials made it to vaccine efficacy testing [13]. These trials tested a number of vaccine components and regimens; using different HIV proteins, vectors and boosting schedules. The first wave of vaccine trials was focused on inducing neutralizing antibodies, which for other infectious diseases has proven successful. Unfortunately, no vaccine to date has been able to elicit broadly neutralizing antibodies that provide protection from HIV transmission. Due to failures in this approach, the second wave of vaccine trials focused on inducing cell-mediated immunity with the hope that a vaccine induced cellular response could block infection. The debate over which of these two approaches should be prioritized continues today. The current phase of vaccine design attempts to induce multiple immune responses with the purpose of potentially providing protection through a combination of responses. To date, out of the scores of trials conducted, the RV144 Thailand HIV vaccine trial is the only trial with any measurable reduction in risk of infection - 31.2% vaccine efficacy [14]. Moderate efficacy is not ideal. However, immune correlate studies from RV144 have provided insights into potential mechanisms of reduced risk of acquisition. RV144 and its subsequent analyses are discussed in great detail throughout this thesis as they shape the field's current foundation for correlates of immunity.

1.2 Mucosal Transmission and the Early Events of HIV Infection

Mucosal transmission of HIV involves a complex yet strikingly inefficient series of events that are not yet fully understood [15, 16]. Much of this complexity is due to the natural variability found in several fundamental characteristics of HIV. The field of HIV research has long sought to understand these variabilities to trace the evolutionary history of the virus, track demographic trends in the epidemic, and more importantly, develop effective therapies and prevention strategies to control the spread of infection.

One key variability in HIV is its vast genetic diversity (Figure 1.1). In order to organize the evolutionary and sequence differences of the major M group of HIV-1, isolates have been classified into subtypes based on phylogenetic similarity of their genome sequences. The major subtype groups are labeled with letters: A1, A2, A3, A4, A6, B, C, D, F1, F2, G, H, J, and K. More complete genome sequencing of the subtypes led to the identification of prevalent inter-subtype recombination events accompanied by the establishment of additional classifications termed circulating recombinant forms (CRFs). These mosaic CRF genomes are labeled with numbers followed by the parental subtype letters that are believed to contribute to their genome (i.e. CRF02_AG, CRF05_DF and CRF01_AE). Each subtype and CRF is characterized by a reference isolate genome sequence and at least three other non-epidemiologically related isolates. The major subtype groups and CRFs often have geographic associations in that some subtypes and CRFs are more prevalent in specific countries or regions than in others. This diversity in sequence across the globe has been a major obstacle to global HIV vaccine design. Some in the field have proposed the potential for subtype- or geographic- specific vaccine development to narrow the scope of protection. However, the feasibility and practicality of this method is still under debate.

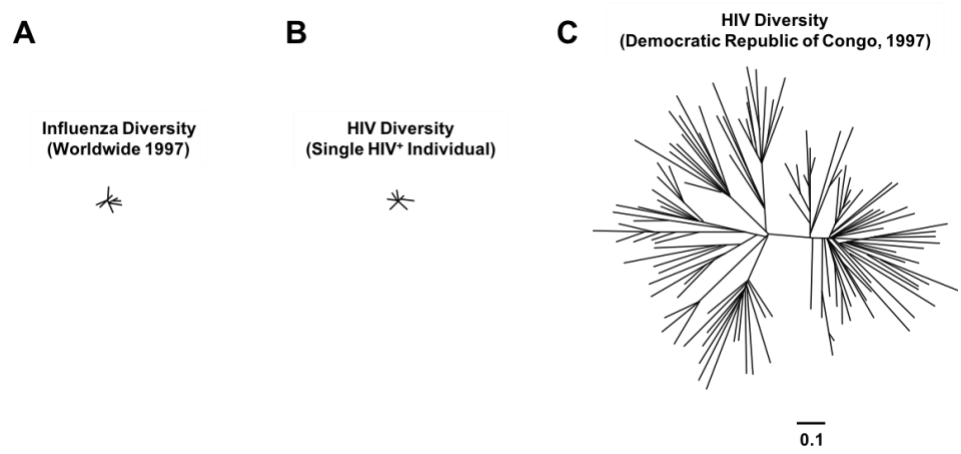

Figure 1.1. Genetic diversity of HIV in an individual and in a country. (**A**) The genetic diversity of HIV isolates replicating in a chronically HIV infected individual as measured by phylogenic analysis of Env sequence is similar to (**B**) the genetic diversity of Influenza replicating worldwide for a given year (1997). (**C**) The genetic diversity of HIV replicating in an entire country during a given year (1997) is several-fold larger than that of an individual. Scale bar is the same for all trees. Adapted from Korber et al. [17].

Another variability that adds to the complexity of HIV transmission involves the genetic diversity within an individual chronically living with HIV. Regardless of HIV subtype, in a chronically HIV infected individual there exists a genetically diverse population of quasi-species of replicating virus. This sequence diversity arises from HIV's error prone reverse transcriptase enzyme (RT), an RNA-dependent DNA polymerase that lacks an effective proofreading mechanism. The high mutation rate of HIV RT, coupled with the virus's rapid replication, is the driver of not only sequence diversity in an infected individual but also the genetic diversity observed in populations across the globe. Additionally, the HIV genome is subject to recombination events, or large insertions and deletions, and of course, significant immune pressure from the host. These agents all add to the complexity in which vast genetic diversity of HIV is achieved.

Despite the existence of multiple genetically diverse isolates replicating in an HIV infected individual, very often, at the time of HIV transmission, only a single or very few

genetically distinct "founder" isolates is able to establish a productive infection in a recipient (Figure 1.2). Studies of natural HIV transmission have shown that despite vast heterogeneity in the donor preceding transmission, extreme sequence restriction leads to nearly homogeneous viral sequences in the recipient. This genetic bottleneck is the result of extreme selective pressure against the virus as it leaves the donor and makes its way through the genital mucosa of the recipient to establish infection. Sequence analyses from transmission events among discordant heterosexual couples shows that in roughly 80% of individuals tested, only a single viral isolate is transmitted [18-20]. SIV and SHIV transmission studies (non-human primate models for HIV) have also shown that infection with a heterogeneous viral stock leads to one or very few isolates establishing infection. That HIV reproducibly goes through cycles of genetic restriction during transmission suggests that the barrier to transmission is quite high and that the efficiency of transmission is low.

The existence of such a severe genetic bottleneck during HIV transmission suggests that transmission of HIV is also extremely inefficient. In fact, studies of monogamous couples in the Rakai district of Uganda that are discordant for HIV show that productive infection of the HIV negative partner occurred from roughly 1 in 1000 coital acts [15]. This study also showed that the risk of HIV transmission is increased during the first 2.5 months after acquisition, when the virus is replicating at extremely high levels [16]. This study and others suggest that while there are high levels of replication throughout the length of HIV infection, barriers exist that make transmission inefficient. This barrier of transmission efficiency is only rarely overcome and is aided by extremely high levels of replicating virus.

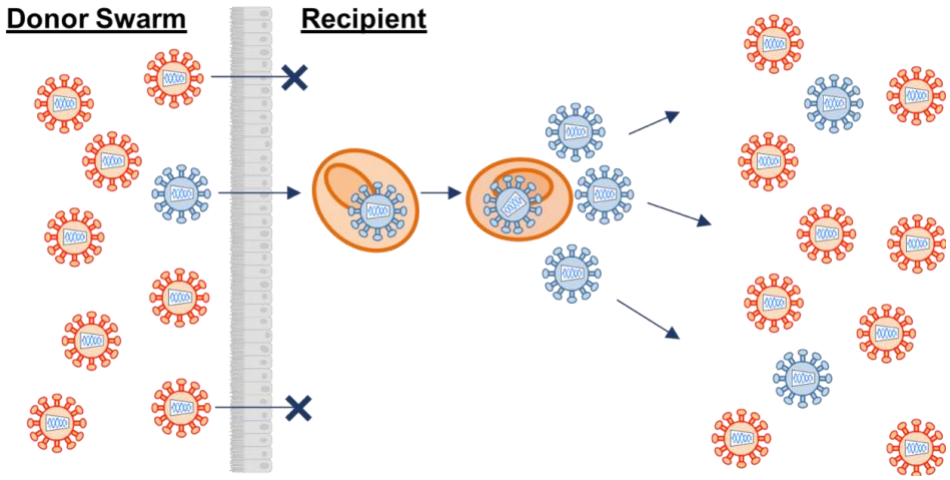

Figure 1.2. Genetic Bottleneck During Mucosal HIV Transmission. In an HIV infected individual, there exists a replicating population of genetically diverse quasi-species of virus. During mucosal transmission, this swarm undergoes extreme restriction crossing the mucosal barrier such that only a single, genetically distinct (Blue virion), or very few isolates, establish a productive infection in the recipient.

Immediately following a transmission event, the virus begins replicating in target cells of the newly infected individual. Clinical characterizations are used to classify the progression of the early stages of HIV infection (Figure 1.3). The first stage following transmission is known as the eclipse phase, where viral RNA and protein are undetectable in the blood. This phase has historically been characterized to last seven to ten days. With the development of ultra-sensitive detection assays, viral RNA can now be detected in blood within the first 3-5 days after transmission. During the eclipse phase, viral RNA levels in the blood grow exponentially due to rapid viral replication in $CD4^+$ T-cells. After viral RNA becomes detectable in the blood, the infection is considered to be in Fiebig stage 1. This stage continues until the viral protein p24 can be detected in blood, which usually occurs around 15 to 18 days after transmission. During this time, the $CD4^+$ T-cell count, which in healthy, uninfected individuals is ~1000 cells/mm^3, declines rapidly to ≤500 cells/mm^3. This destruction of immune cells leads to flu-like symptoms (fever, chills, fatigue, headache, sore throat, etc.), although not in all cases. Fiebig stage 3 follows and is

characterized by detection of anti-HIV antibodies in the blood. The detection of antiviral antibodies in the blood indicates that the immune system is attempting to combat the infection through adaptive immune pressure. As immune pressure builds against the transmitting isolate early in infection, selection for minor variants or newly acquired mutations takes place. This game of 'cat and mouse' in which the immune system is chasing a rapid, ever-changing target while the virus is depleting a significant fraction of immune cells leads to the destruction of critical host immune barriers and the eventual development of AIDS.

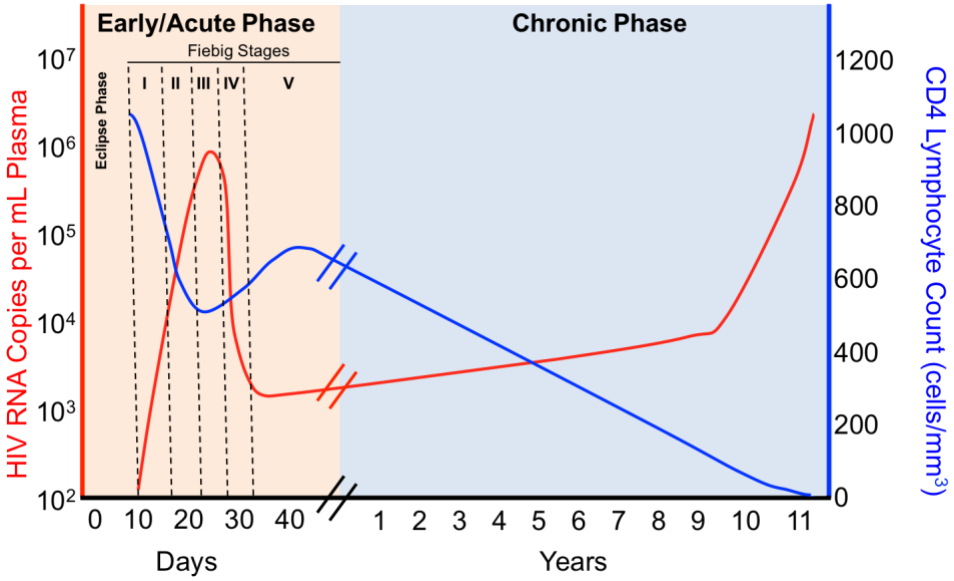

Figure 1.3. Stages of HIV infection classified by viremia and CD4$^+$ T-cell count. Schematic of viral load (left y-axis) and CD4 lymphocyte count (right y-axis) over time (x-axis) of HIV infection. The early/acute phase of infection is classified into stages of clinical diagnostics (Fiebig stages).

Acute HIV infection is characterized by significant replication in and subsequent depletion of CD4$^+$ T-cells, particularly of the gut associated lymphoid tissue (GALT). The GALT has the largest surface area of all organs, and because the GALT comes into direct contact with a hostile external environment, it therefore contains a large population of protective immune cells. The GALT harbors some ~50% of the body's CD4$^+$ T-cell

population. During HIV infection, the $CD4^+$ T-cell population can be cut in half as a result of viral replication and targeted cell killing. Irreversible damage to the GALT is associated with the loss of mucosal barrier maintenance that protects the gut tissue from pathogens, as well as the digestive and metabolic functions of the gut. This gut-tropic nature of HIV infection is not completely understood, although this topic is introduced in section 1.5 further and discussed throughout this thesis.

While HIV can enter both activated and nonactivated target cells, the virus requires cellular activation for high levels of replication to take place. Highly activated cells are therefore ideal targets for HIV infection. A major barrier for transmission of HIV is a low frequency of activated target cells in the genital mucosa. Only ~20% of total human $CD4^+$ T-cells are activated [21]. In *Rhesus macaques,* the majority of the $CD4^+$ T-cells in the vaginal and ecto- and endo-cervial regions are of a resting phenotype. There are reportedly 70 times more resting $CD4^+$ T-cells than activated $CD4^+$ T-cells in this compartment [22]. While these resting target cells can become infected, they tend to be self-limiting and produce small foci of founder viral populations in SIV models. Therefore, the low frequency of activated $CD4^+$ target cells in the genital tract contributes to the inefficiency of HIV transmission.

In addition to $CD4^+$ T-cells, there is also an abundance of $CD4^+CCR5^+$ macrophages and dendritic cells in the genital mucosa. These cells are believed to play a major role in the early events of a productive HIV infection. Dendritic cells and Langerhan cells are capable of capturing virions and/or becoming infected [23-25]. DC-SIGN, a C-type lectin receptor expressed on dendritic cells, is capable of binding HIV virions. DC-SIGN captured virions can then be passed to target $CD4^+$ T-cells [26]. These cells may also

play a role in trafficking of virions from the genital compartment to the gut mucosa [27], although the significance of these findings in the context of HIV transmission is not entirely clear.

Another subset of cells that is believed to play a major role in the early events of transmission are Th17 cells. During acute HIV and SIV infection, there appears to be a preferential depletion of Th17 cells of the GALT, suggesting they are targets of robust viral replication [28-30]. The frequency of Th17 cells in the gut was shown to negatively correlate with plasma HIV or SIV load [31]. A loss of Th17 cells also correlated with SIV disease progression [32]. Additionally, a majority of Th17 cells express high levels of integrin $\alpha_4\beta_7$, a lymphocyte gut homing receptor [33] described in great detail throughout this thesis.

1.3 The HIV-1 Envelope Protein

HIV is an enveloped retrovirus with a positive-sense single-stranded RNA genome. The HIV genome contains nine overlapping genes translated in multiple reading frames that are alternatively spliced to generate 15 proteins. The genome inside an HIV particle is protected by a viral capsid shell, which in turn is surrounded by the viral envelope (Figure 1.4). The viral envelope is composed of the lipid membrane of the host cell of which the virion budded. This lipid membrane is studded with spikes of HIV envelope glycoprotein (Env), as well as host cellular receptors that were transiently or actively incorporated into the budding virion. Env on virions interacts with host receptor CD4 and coreceptors CCR5 and CXCR4 to gain entry into target cells. In this way, Env is critical for viral entry and infection.

The HIV Env protein is encoded by a roughly 2,550 base pair *env* gene that when synthesized into amino acid, encodes three distinct proteins. The Env propeptide is ~850 amino acids (aa) long. The first segment encodes the signal peptide (SP) of the HIV Env protein which is ~30 aa in length. Much discussion of the SP is conducted below and is a major topic of research throughout this thesis. The SP is post-translationally cleaved resulting in a ~820 aa precursor protein known as gp160. The gp160 precursor protein is further processed into two functional proteins gp120 (~480 aa) and gp41 (~340 aa). The gp120 and gp41 proteins non-covalently bind to form heterodimers. These dimers in turn form trimers known as Env trimer spikes. The spikes are transported to the surface of infected cells, and are incorporated into budding virions with the help of the viral protein Gag.

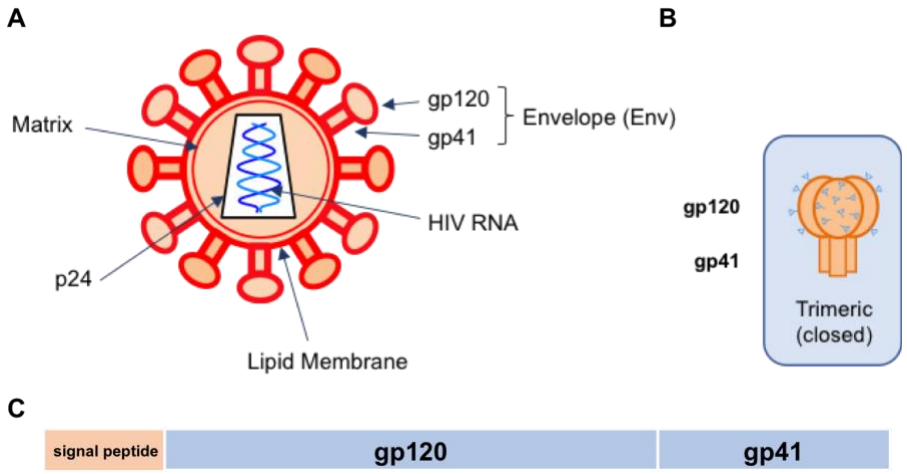

Figure 1.4. The HIV envelope proteins gp120 and gp41 coat HIV particles. (**A**) Schematic of an HIV particle coated with Env spikes. (**B**) The Env proteins gp120 and g41 associate non-covalently to form heterodimers. These dimers form trimers that make up the Env spike. (**C**) The HIV *env* gene encodes a signal peptide, gp120 and gp41. This is translated as one propeptide which is processed into mature Env proteins.

Each of the two functional Env proteins is further characterized into functional or structural domains. The gp120 protein is divided into sequentially numbered conserved (denoted as C) and variable (denoted as V) domains: C1, V1, V2, C2, V3, C3, V4, C4, V5,

C5. These domains or loops of gp120 are created during the post-translational processing of Env that is discussed in detail in the next section and a topic of research included in this thesis. The gp41 protein is divided into the fusion peptide domain (FP), the helical N-terminal heptad repeat (NHR), the C-terminal heptad repeat (CHR) and the trans-membrane domain (TM).

Viral entry into a host cell is accomplished by interactions between the Env spike and host cellular receptors. The main target cells of HIV are $CD4^+$ T-cells and CD4 is the primary receptor for HIV. A hallmark of HIV disease is a substantial depletion of $CD4^+$ T-cells in the peripheral blood as well as in the gut associated lymphoid tissues (GALT) as a result of the infection and destruction of these cells. The loss of these critical $CD4^+$ immune cells is also attributed to the compromised immune system that characterizes the progression of HIV disease into AIDS.

The Env spike on HIV targets $CD4^+$ T-cells for infection by binding directly to CD4 through a discontinuous epitope on gp120. CD4 binding to gp120 causes a conformational shift in Env revealing an otherwise inaccessible co-receptor binding site on gp120. This conformational shift allows for efficient co-receptor binding to the co-receptor binding site. There are two major co-receptors for HIV entry, CCR5 and CXCR4. Upon co-receptor binding to Env, gp41 undergoes extensive rearrangement revealing the FP domain of gp41, and brings the viral membrane in close proximity to the target cell membrane. The FP domain of gp41 is then inserted into the membrane of the target cell allowing for fusion of the two membranes to occur.

Due to the expression of Env on virion surfaces and the role of Env in viral entry, much work has been done in the characterization of Env epitopes with the aim of targeting

key Env regions for preventative and therapeutic strategies. In this regard, many monoclonal antibodies (mAbs) have been isolated from immunized and HIV/SIV/SHIV infected animals, as well as HIV infected and immunized humans. These mAbs target several epitopes of Env with varying degrees of neutralizing capacity. Major sites of neutralizing antibodies are: the V1/V2 loop, the CD4 binding site, the V3 glycan patch, the gp120/gp41 interface and the membrane-proximal external region (MPER) located in the gp41 ectodomain adjacent to the viral membrane.

Crystal structures of various Env-specific mAbs with recombinant Env proteins and peptides have been solved. This structural data provides a snapshot of the structures and conformations in which Env can adopt and present. These crystal structures have determined that Env is exceptionally fluid in its structure in that several domains of gp120 can adopt multiple distinct conformations. This is particularly true for the V1V2 loop of gp120, which is a major topic of this thesis.

As mentioned previously, the only HIV vaccine trial to elicit any protective immune response was the RV144 Thailand HIV vaccine trial. This trial provided only moderate protection from HIV infection. The vaccine consisted of a DNA prime, protein boost regimen via four doses over 24 weeks. The prime, ALVAC-HIV (vCP1521), consisted of an inert form of canary pox virus vector. This vector is derived from an avian virus and does not cause disease or replicate in humans. It has previously been used in cancer vaccine candidates. The canary pox vectored prime contained genetically engineered versions of the *gag, pol* and *env* genes of HIV. The Env included in this vector expresses the 92TH023 isolate gp120 (subtype E) linked to the LAI isolate gp41 (subtype B). HIV subtype A/E, where the Env sequence is from the ancestral E subtype, is the predominant circulating

subtype in Thailand. The prime was given at weeks 0 and 4, and was intended to induce cellular immunity to HIV. At weeks 12 and 24, the ALVAC prime was given in addition to an AIDSVAX B/E boost. The AIDSVAX boost consisted of recombinant gp120 proteins from subtype E (CM244; A244) and subtype B (MN). This protein boost was intended to induce a neutralizing antibody response to Env. The trial enrolled over 16,000 volunteers, half of which received the complete vaccine regimen, while the other half was administered a placebo. The recipients were followed for three years for vaccine safety and efficacy as well as immune response analysis.

The observed vaccine efficacy for RV144 was 31.2% [14]. The surprising finding of moderate vaccine efficacy initiated a global interest in identifying potential mechanisms of protection and immune correlates of reduced risk of acquisition. Analysis of samples obtained 2 weeks after the final immunization of 205 uninfected vaccinees and 41 vaccinees who became infected identified the binding of IgG Abs to the V1V2 region of Env correlated inversely with the rate of infection [34]. Researchers also found that neutralizing antibodies did not correlate with the risk of infection. In fact, while RV144 was able to induce long lasting memory responses in a subset of vaccine recipients, the vaccine regimen did not induce bNabs [35]. Further sequence analysis of breakthrough viruses identified positions 169 and 181 in the V2 region of Env as the targets of vaccine induced immune pressure [36]. This finding suggests that vaccine-induced V2 responses played a role in the partial protection conferred by the RV144 vaccine regimen. The significance of these two residues in the V2, the way in which they are presented structurally, and their potential role in HIV disease is a major topic of this thesis.

1.4 HIV-1 Env Post-translational Processing

The Env proteins gp120 and gp41 are translated as a single gp160 propeptide from the *env* gene in the HIV genome as introduced in the preceding section. Much like all other secreted or transmembrane proteins, the Env propeptide contains a signal peptide (SP) (Figure 1.4). The SP of Env is unusually long (30aa) and is post-translationally cleaved from gp160. The gp160 protomer is further processed by cleavage into separate gp120 and gp41 proteins. These proteins non-covalently form dimers. The gp120/gp41 dimers in turn form trimers that decorate mature virions. However, much is done in the way of post-translational processing before the Env proteins are incorporated into mature virions.

Translation of the Env transcript into an amino acid chain is initiated by a ribosome in the cellular cytosol (Figure 1.5). The first amino acids of Env to emerge from the exit tunnel of the translating ribosome contains the SP. Once the SP has emerged, it is bound by a ribonucleoprotein complex known as the Signal Recognition Particle (SRP). Binding of the SRP to the SP causes a pause in protein synthesis known as elongation arrest, giving time for the SRP-SP-ribosome-mRNA complex to traffic to the Endoplasmic Reticulum (ER) membrane. This trafficking is achieved by SRP binding to its ER-membrane-embedded SRP receptor (SRPr). Once docked on the SRPr, the ribosome-mRNA-SP complex is transferred to the ER channel protein called the translocon complex, and the SRP-SRPr complex dissociates. The SP is inserted into the pore of the translocon, and hydrophobic residues in the SP mediate its incorporation into the membrane of the ER. Because the SRP is dissociated from the translation complex, translation of the Env protein continues. As the remaining Env mRNA transcript is translated, the newly synthesized amino acid chain traverses the pore of the translocon by Brownian ratchet, and the nascent

peptide emerges inside the lumen of the ER. The gp41 transmembrane (TM) domain contains a membrane anchor known as a stop-transfer signal. This signal is embedded into the ER membrane preventing gp160 from being fully released into the ER lumen and presenting Env in the correct orientation for expression on the surface of the cell. The SP is then post-translationally cleaved by an ER resident signal peptidase and the gp160 continues on the maturation pathway.

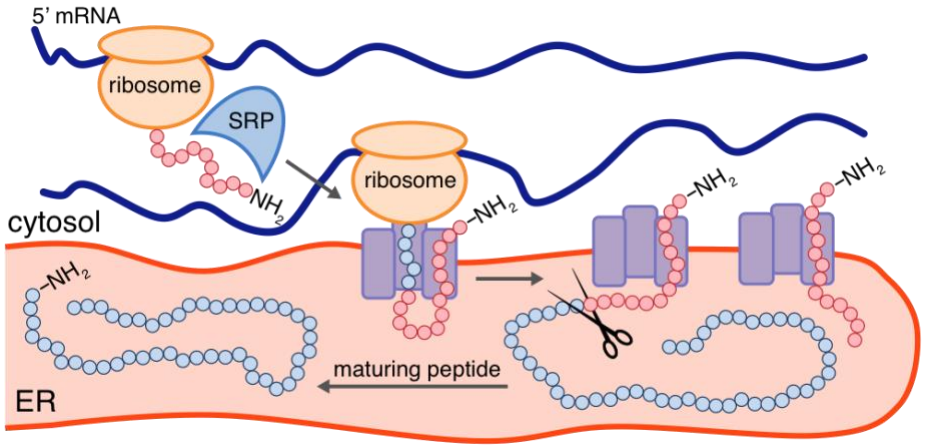

Figure 1.5. The role of the signal peptide in early- and post-translational processing. The SP of HIV Env is the first domain of the Env polypeptide to be translated and exits the translating ribosome first. Attachment of the newly synthesized SP by a signal recognition particle (SRP) targets the peptide-translating ribosome to the translocon pore of the ER. Once at the translocon of the ER, translation of the remaining polypeptide continues into the ER lumen. The SP of gp120 is cleaved post-translationally as initial post-translational modifications are made.

Even before the SP is cleaved from gp160, glycans are added to the nascent propeptide. As with all N-linked glycosylation, the initial glycan structure, $Glc_3Man_9(GlcNAc)_2$, is added to the asparagine of N-linked glycosylation motifs (NxS/T, where x is any amino acid except proline). This takes place in the ER by a resident glycosyltransferase. The terminal Glc_3Man_9 glycan structure is extensively modified by ER glycosidases and glycosyltransferases, which sequentially remove terminal mannose (Man) residues and add other glycans (Figure 1.6).

HIV Env is one of the most glycosylated proteins known to man. In fact, half of the molecular weight of the HIV glycoprotein is contributed by glycans. One hypothesis for the excessive glycosylation of Env is to shield critical epitopes on the protein from neutralizing antibodies. HIV gp120 has on average 25 N-linked glycosylation sites but this number ranges widely from 18 to 33 (Figure 1.6). Many of these N-linked glycosylation sites are located in the variable loops of gp120, while some are located in the core of gp120 and to a lesser extent in gp41.

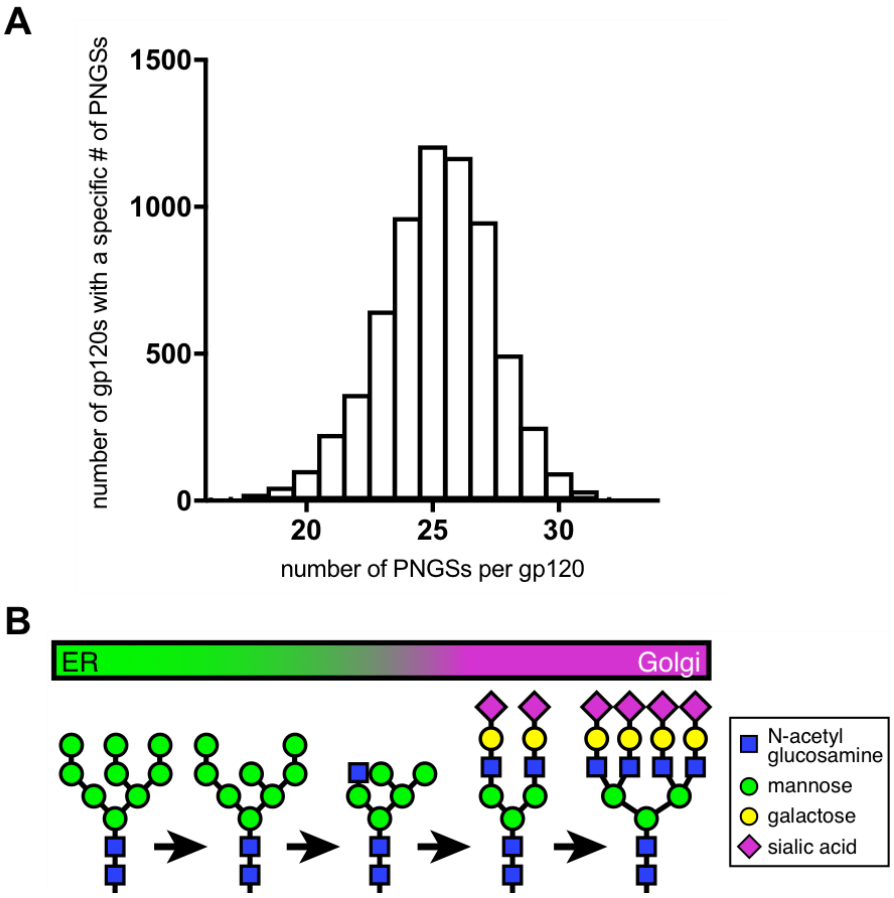

Figure 1.6. Glycosylation of the HIV Envelope protein. (**A**) Histogram of the number of potential N-linked glycosylation sites (PNGSs) of 6635 gp120 sequences from the Los Alamos HIV Sequence Database. (**B**) N-linked glycans are added as high mannose structures in the ER which are subsequently trimmed and modified into more complex carbohydrate glycans as the glycoprotein traverses the ER-Golgi network.

While still in the ER, and with the assistance of resident chaperone proteins like calnexin (whose interaction with gp160 is modulated by the Env SP), immature gp160 begins to fold into its native structure. The importance, and complexity, of N-linked glycosylation in Env folding, structure and function has been extensively studied. The glycosylation inhibitor, tunicamycin, disrupts the cleavage of the SP from gp160 due to aberrant folding which leads to retention in the ER. Disruption of N-linked glycosylation motifs by direct mutation have shown that key glycosylation sites are necessary for the correct folding of gp120. In addition, glycosylation inhibitors reduce the secretion of recombinant gp120 into cellular supernatants while retaining immature gp120 in cell lysates. Cells transfected with Env expression constructs do not produce gp120 capable of binding CD4 in the presence of several such glycosylation inhibitors. These data show that glycosylation is necessary for the correct folding of gp120.

After initial folding is complete in the ER, oligomerization of gp160 begins and Env trimer spikes are formed. Studies have shown that mutating asparagine residues in the core of gp120 to structurally similar glutamines or alanines, results in misfolding and a reduction in trimer formation. This suggests that at least some glycosylation is required for the correct folding and trimerization of gp160.

In the Golgi, gp160 is proteolytically cleaved by the cellular protease, furin [37] at a highly conserved K/R-X-K/R-R motif [38, 39]. Non-covalent interactions maintain the association of gp120 and gp41. However, Env is rapidly recycled from the surface of the cell by endocytosis [40, 41] and some gp120 is shed. The internalization and shedding of gp120 result in low levels of Env incorporation into virions (~10 Env spikes/virion) [42]. While Env trimer spikes are incorporated into virions and involved in viral entry,

incompletely-processed or non-trimer associated Env are also incorporated. The relative abundance of this immature Env in the context of virions is not well understood, nor is its role.

1.5 The Connections Between HIV and $\alpha_4\beta_7$

Our laboratory has extensively reported on an intriguing interaction between the HIV Env protein gp120 and the integrin $\alpha_4\beta_7$ ($\alpha_4\beta_7$) on lymphocytes [43-45]. Env gp120 not only binds to $\alpha_4\beta_7$, but it also provides a signal to the cell through $\alpha_4\beta_7$ leading to activation and the expression of LFA-1. This interaction appears to have evolved in mimicry of the interaction between $\alpha_4\beta_7$ and its natural ligand, mucosal vascular addressin cell adhesion molecule 1 (MAdCAM). Integrin $\alpha_4\beta_7$ is expressed on T-, B-, and NK- cells. MAdCAM is constitutively expressed on the high endothelia venules (HEV) in Peyer's patches (PP) that line the gut. The binding of $\alpha_4\beta_7$ to MAdCAM is a critical step in the lymphocyte multistage adherence cascade: tether, roll, activate, adhere, and then extravasate through the endothelia layer to the tissue compartment (Figure 1.7). Because of the gut-tropic expression of MAdCAM and the nature of its interaction with $\alpha_4\beta_7$, the integrin is known as the gut-homing receptor. The finding that HIV has adapted the ability to interact with the gut-homing receptor, integrin $\alpha_4\beta_7$, paired with the gut-tropic nature of transmission and acute infection of HIV, makes for an implicit connection and compelling story.

In healthy humans, $\alpha_4\beta_7$ plays two biological roles 1) aiding in lymphocyte cell migration and tissue homing and 2) providing a secondary, costimulatory signal to T-cells. Regarding the first function, $\alpha_4\beta_7$ maintains the unique capacity to mediate lymphocyte

homing to the GALT, a major site of HIV replication. This process of migrating and homing to the gut is highly ordered. Migrating lymphocytes face extreme conditions in the shear force and high pressure that is present in the microvenules in which they travel. Therefore, overcoming this stress is a significant challenge. To mitigate these conditions, an array of adhesion molecules, including $\alpha_4\beta_7$, on the surface of migrating cells as well as the cells lining the endothelial vessels participate in a multistep adhesion cascade. The first step of this cascade involves the tethering and rolling of lymphocytes to the endothelial layer lining the vessels. This is mediated by L-selectin on lymphocytes binding to sialyl-Lewis structures that decorate the glycans of endothelia receptors involved in this stage. MAdCAM contains sialyl-Lewis structures involved in binding L-selectins during this step. This interaction mediates the rolling of the lymphocyte along the vessel wall. Integrin $\alpha_4\beta_7$ in its intermediate state and its ligand MAdCAM are also involved in this tethering and rolling step [46, 47]. The initial rolling and tethering interaction to the endothelial vessels activates the second step in the cascade: signaling by chemokines. The release of chemokines induces the activation of $\alpha_4\beta_7$ to the extended, open conformation. Other integrins including $\alpha_4\beta_1$ and LFA-1 are also activated in this step. In the third phase, firm arrest of lymphocytes takes place through strong adhesive interactions between lymphocyte integrins and their ligands on endothelial cells. Extravasation through the endothelial layer into the tissue is the final step of this cascade. The $\alpha_4\beta_7$ -MAdCAM interaction is involved in the rolling adhesion and firm arrest steps of the cascade. This is critical for $\alpha_4\beta_7^+$ T-cell homing to the lymph nodes and PP of the GALT.

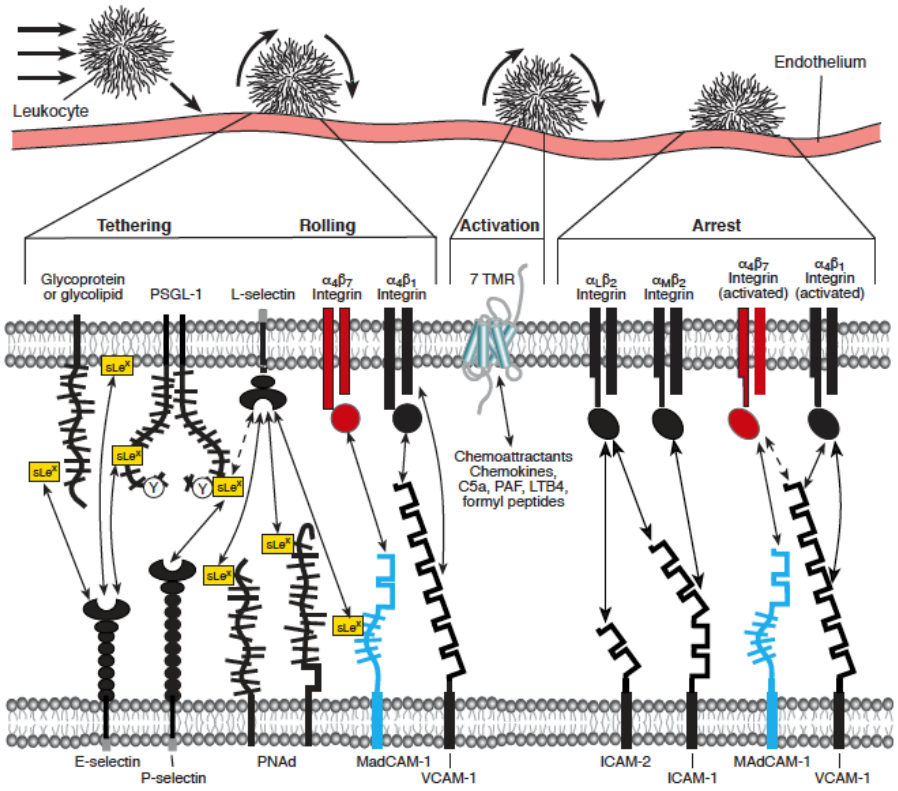

Figure 1.7. The role of $\alpha_4\beta_7$ in the lymphocyte multistep adhesion cascade. A schematic depicting lymphocyte tethering, rolling, activation and firm-arrest along the endothelial cells of a microvenule. The cascade begins with low affinity tethering of lymphocyte receptors to selectins on the endothelial cell. This tethering leads to rolling of the lymphocyte which involves $\alpha_4\beta_7$ and MAdCAM. This is followed by subsequent activation of the lymphocyte. After activation of the lymphocyte, the active, extended conformation of $\alpha_4\beta_7$ interacts with high affinity to MAdCAM on the endothelial membrane causing firm arrest. Integrin activation and increased affinity for MAdCAM and VCAM are critical for the cell arrest that precedes extravasation into the tissue.

As alluded, integrin $\alpha_4\beta_7$ can adopt at least three distinct conformations: bent (inactive), extended (intermediate), and open extended (active). The intermediate conformation of $\alpha_4\beta_7$ is associated with low affinity binding to MAdCAM while the open conformation is associated with higher affinity binding to MAdCAM. The active state of $\alpha_4\beta_7$ is regulated by intracellular signaling that modulates its conformation. During the multistage adhesion cascade, the extended/intermediate conformation mediates rolling

adhesion to MAdCAM while the open extended/active conformation mediates firm adhesion.

Integrin $\alpha_4\beta_7$ binds to an LDT tripeptide motif in its natural ligand, MAdCAM, in a divalent cation-dependent manner. The strength of the interaction between $\alpha_4\beta_7$ and MAdCAM can be modulated by manipulating the cation concentration. The interaction is highest in the presence of Mn^{++} and weakest in the presence of Ca^{++}. The interaction has intermediate strength in the presence of Mg^{++} and has no binding in the presence of EDTA, a metal cation-chelating agent. It is the coordination of a divalent cation in the metal ion dependent adhesion site (MIDAS) of β_7 that bridges the aspartic acid of the MAdCAM LDT motif to $\alpha_4\beta_7$. Because the MIDAS metal ion identity impacts the coordination geometry, using the different divalent cations mentioned above can modulate the affinity of the interaction.

Integrin $\alpha_4\beta_7$ binds to a highly conserved LDV/I motif at positions 179-181 in the V2 loop of gp120. This interaction is also divalent cation-dependent. The binding of gp120 to $\alpha_4\beta_7$ is inhibited by MAdCAM and small molecule mimetics of the LDV/T motif. Subsequent studies have identified a second, more cryptic, motif that is involved in the interaction between $\alpha_4\beta_7$ and gp120 slightly upstream of the LDV that includes the residues QKE at position 170-172 [45].

In vitro, $CD4^+$ T-cells that express high levels of $\alpha_4\beta_7$ are preferentially infected by HIV and later depleted [44], presumably due to the cell death associated with cellular infection. The CD4 receptor has been shown by immunofluorescence and FRET imaging to co-localize with $\alpha_4\beta_7$ as well as CCR5, an entry co-receptor, on $CD4^+$ T-cells activated with gp120. Unlike CD4 and CCR5, however, $\alpha_4\beta_7$ is not an entry receptor nor is it required

for infection *in vitro*. That this CD4-CCR5-$\alpha_4\beta_7$ complex is presented together on ideal target cells for HIV infection, suggests a role for $\alpha_4\beta_7$ in HIV infection, although this role has yet to be determined.

Studies using a non-human primate model of SIV transmission support a role for $\alpha_4\beta_7$ in the establishment of SIV infection in the GALT [33, 48, 49]. In collaboration with Professor Aftab Ansari and others at Emory University, our group has antagonized the interaction between $\alpha_4\beta_7$ and gp120 in nonhuman primate models of SIV transmission and acute infection. Pre-infusion of female rhesus macaques with an anti-$\alpha_4\beta_7$ mAb challenged intravaginally with a weekly low-dose of pathogenic SIV_{mac251} lead to significant protection of half the treated animals, and a profound delay in infection of the animals that became infected [50]. Of note, the animals that received infusions of the anti-$\alpha_4\beta_7$ mAb and became infected showed protection of $CD4^+$ T-cells in the gut.

An additional study was conducted to look at the impact of antagonizing $\alpha_4\beta_7$ in the context of acute SIV infection. Rhesus macaques were infected with a high dose of pathogenic SIV_{mac239} and then placed on daily ART. After 4 weeks of ART, the animals were put on the same anti-$\alpha_4\beta_7$ mAb infusions used in the study above. At week 18, ART was discontinued while Ab infusions continued. As expected, after termination of ART, the control animals had significant viral rebound. However, all 8 of the $\alpha_4\beta_7$ treated group experienced either significantly lower viral rebound or no rebound at all. All 8 animals went on to control viremia to below detectable levels without any therapy [51]. During the phase of this study where the animals were undergoing dual therapy (simultaneous ART and $\alpha_4\beta_7$ Ab administration), there was a rebound of the $CD4^+$ T-cell population of the gut in the $\alpha_4\beta_7$ mAb treated animals. The control animals never recovered their gut $CD4^+$ T-

cell population. Additionally, while all animals in the study had similar gp120-reactive sera, 8/8 of the $\alpha_4\beta_7$ mAb treated animals but only 3/7 of control animals had sera that reacted against a cV2 peptide. This suggests that the $\alpha_4\beta_7$ mAb treatment promoted a V2 antibody response that is similar to the anti-V2 response observed in the RV144 vaccine trial.

$\alpha_4\beta_7^+$/CCR5$^+$/CD4$^+$ memory T-cells are found in the rectal and vaginal mucosa where initial infection of cells is likely to occur during mucosal transmission [21, 44]. Several studies have shown that $\alpha_4\beta_7$ mediates the trafficking of these potential target cells from the genital tract to mesenteric lymphnodes (MLNs) [52-54]. CD4$^+$ T-cells are believed to be among the very first cells infected early in mucosal transmission [22, 26], however, it is not known whether they are also $\alpha_4\beta_7^+$.

In humans, $\alpha_4\beta_7$ expression on CD4$^+$ T-cells is associated with cellular activation in that $\alpha_4\beta_7^{high}$CD4$^+$ T-cells also express the T-cell activation markers CCR5, CD69, and Ki-67 [21, 44]. Because of HIV's inefficiency in transmission, infecting activated cells likely represents a key step in establishing a productive infection. This likely contributes to the finding that HIV preferentially infects $\alpha_4\beta_7^+$/CD4$^+$ T-cells *in vitro*. This also promotes the hypothesis that HIV evolved a specific affinity for $\alpha_4\beta_7$ as a means of targeting ideally activated, highly susceptible cells with gut-homing potential. In this way, HIV would increase the likelihood of trafficking to the gut where a high proportion of target CD4$^+$CCR5$^+$ T-cells are located.

On isolated human CD4$^+$ T-cells, roughly half of the cells express medium levels of $\alpha_4\beta_7$, while the other half expressed low levels of $\alpha_4\beta_7$. In the MLN and PP, specialized DCs convert vitamin A into retinoic acid (RA). RA acts directly on T-cells to induce a gut-

homing phenotype that includes increased $\alpha_4\beta_7$ expression [43]. This gut homing phenotype can be recapitulated *in vitro* by culturing peripheral blood mononuclear cells (PBMCs) in the presence of RA. We have shown that PBMCs cultured in RA have higher levels of $\alpha_4\beta_7$ and that these cells bind gp120 with greater efficiency that non-RA treated PBMCs.

Our group has also shown that gp120 binding to $\alpha_4\beta_7$ can induce intracellular signaling that leads to cellular activation. We originally showed that gp120 can activate the expression of LFA-1 [43]. While HIV virions are sufficient for infection, HIV can spread more efficiently through cell-to-cell transfer of HIV. This is accomplished through the formation and stabilization of a virological synapse (VS). Similar to an immunological synapse, a VS is a tight adhesive junction formed between an infected cell and an uninfected target cell, across which virus can be transferred efficiently. LFA-1 has been shown to play a role in the stabilization of the VS. As mentioned above, gp120 can activate LFA-1 expression suggesting a role for $\alpha_4\beta_7$ in facilitating cell-to-cell spread of HIV. The additional observation that $\alpha_4\beta_7$ colocalizes in clusters with CD4 and CCR5 on the surface of gp120 stimulated cells, further supports the hypothesis that $\alpha_4\beta_7$ plays a role in HIV infection and cell-to-cell spread.

1.6 Thesis Objectives

1.6.1 Overview

Each section detailed above describes a distinct subject that has received incredible attention in the global efforts to prevent HIV transmission and treat HIV disease progression. We believe that all of these subjects – mucosal transmission of HIV, the post-

translational processing of HIV Env and the interaction of gp120 with $\alpha_4\beta_7$ – are connected in a fundamental way. Genetic signatures of HIV transmission modulate gp120 post-translational processing which significantly impacts the structure of gp120 in a way that influences its interaction with its receptors including $\alpha_4\beta_7$. Studying such genetic signatures will shed light on events surrounding transmission and acute HIV infection. It is our hope that the information presented as part of this thesis supplements the understanding of HIV transmission, and contributes in a serious way to the field's efforts of developing a robustly protective HIV vaccine.

1.6.2 Objectives

To further our understanding of the impact of protein processing on HIV Env structure and function in the context of HIV transmission, I have advanced several research projects to address shortcomings in these areas individually and make connections between them.

In chapter 2, I present work examining the role of the HIV Env SP in influencing key characteristics of gp120 structure and function. We show that the SP alone can influence the glycosylation pattern of recombinant gp120 proteins in a significant way that alters its interaction with transmission-relevant host receptors. We also show that the altered glycosylation profile observed for these recombinant proteins mediated by the SP have major influence on the antigenicity of the gp120s. I go on to show that, in the context of infectious molecular clones, the SP can impact the neutralization sensitivity of virions to anti-V2 domain neutralizing antibodies. These studies make the connection between genetic signatures of transmission and potential structural or functional targets that, due to selective pressure, provide some isolates with increased transmission fitness.

In chapter 3, I describe the epitope of an interesting class of weakly neutralizing anti-gp120 V2 domain mAbs that inhibit the adhesion of $\alpha_4\beta_7$ and gp120. These antibodies recognize a helix/coiled conformation of the V2 loop that I show is present on incompletely processed Env and is also present on mature virions. Some of these mAbs were isolated RV144 vaccine recipients. That this epitope, which is precluded from the stabilized Env trimer and is associated with ER-Env, interacts with $\alpha_4\beta_7$ in an overlapping epitope with the RV144 sieve sites brings the intriguing possibility that the weakly neutralizing Ab response of RV144 may be connected to this interaction.

In chapter 4, I describe the links between the influence of genetic signatures of transmission on glycosylation of gp120 discussed in chapter 2 and the role of glycosylation and structure of the V2 interaction with $\alpha_4\beta_7$ in chapter 3. I discuss their connections in the context of HIV transmission and early infection. Also discussed are the questions that still remain in our efforts to understand the early events of HIV transmission.

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Chapter 2:

THE HIV ENV SIGNAL PEPTIDE IMPACTS VIRAL NEUTRALIZATION THROUGH ALTERED GLYCOSYLATION AND ANTIGENICITY OF gp120

2.1 Abstract

The HIV-1 Envelope protein (Env) of early-replicating viruses encode several distinct transmission signatures. One such signature involves a reduced number of potential N-linked glycosylation sites. This transmission signature underscores the importance of post-translational modifications in the fitness of early-replicating isolates. An additional signature in Env involves the over-representation of basic amino acid residues at a specific position in the Env signal peptide (SP). We investigated the potential impact of this SP signature on gp120 glycosylation, antigenicity and viral neutralization. To this end, two recombinant gp120s were constructed, one derived from an isolate that lacks this signature and a second from an early-replicating isolate that includes this signature. Chimeric gp120s were also constructed in which the two SPs were swapped between the isolates. All four gp120s were probed with glycan-, structure- and receptor- specific probes in a surface plasmon resonance binding assay. We found that the SP of Env influences qualitative aspects of Env glycosylation that in turn affect the antigenicity of Env in a major way. The SP impacts the affinity of Env for DC-SIGN, a lectin receptor expressed on dendritic cells that is believed to play a role in mucosal transmission. Additionally, affinity for the monoclonal antibodies 17b and A32, which recognize a CD4-induced, open conformation of Env is also altered. To investigate the impact of SP variation in the context of whole virus, we generated chimeric infectious molecular clones (IMCs) whereby the Env SP of the viral backbone was individually swapped with 11 alternate SPs from diverse isolates. We found that the SP of these IMCs significantly impacted neutralization by anti-gp120 V2 domain mAbs. These results demonstrate that natural variation in the SP of HIV Env can significantly impact the antigenicity of mature gp120 and influence characteristics of

viral neutralization. Thus, the SP is likely subject to antibody-mediated immune pressure and should be included in the design of HIV prevention strategies.

2.2 Introduction

Transmission of HIV across mucosal tissues involves a complex, yet strikingly inefficient, series of events that are not fully understood [1, 2]. It has been shown that among the quasi-species of virus replicating in an infected, sexually transmitting person, very often, only a single isolate establishes infection in the recipient partner [3, 4]. Studies suggest that this genetic bottleneck is not stochastic [3, 4]. Rather, specific structural features of the Env appear to provide transmitting isolates with increased transmission-fitness [5, 6]. The transmitting virus diversifies over time in infected subjects [7] due to random mutations introduced by error prone replication, which are then subjected to immune selective pressure.

The HIV Env is extensively glycosylated, with up to 50% of the mass of gp120 comprised of glycans [8]. However, the number of potential N-linked glycosylation sites (PNGSs) varies significantly between isolates, with a range of 18 to 33 and a median of 25 [9, 10]. Genetic signatures in transmitting HIV isolates have been linked to Env [11, 12]. The best-characterized signature involves a reduced number of PNGSs in transmitting isolates [12]. In addition, shorter variable loops in gp120 have been correlated with this decrease in PNGSs [13]. Mass spectrometry analysis of the glycan profiles of Env from transmitting viral isolates in acute infection shows increased high-mannose residues compared to the glycan profile of Env from chronic phase isolates further suggesting a link between Env glycan content and transmission [14]. It is currently unknown by what precise mechanisms these alterations in glycosylation influence transmission-fitness. One possibility is that altered glycosylation can directly influence the interaction of Env with host cell surface receptors [15], which in turn increase infectivity. Another widely accepted

hypothesis is that once infection is established, the appearance of new PNGSs may shield critical epitopes from neutralizing antibodies that evolve during the course of infection.

A second genetic signature, identified in subtype B isolates, involves an over-representation of basic amino acids, particularly histidine, at position 12 of the Env signal peptide (SP) of transmitting viral isolates (Figure 2.1*)*, which is lost in chronic phase viral isolates [11, 16]. The presence of a signature in this region of the Env SP of transmitting viral isolates has also been confirmed in SIV and SHIV transmission studies [17]. The SP, which lies at the N-terminus of the amino acid sequence of proteins destined for transport to the cell surface or for secretion, directs these nascent peptides to the endoplasmic reticulum (ER) where translation continues, coincident with protein processing (Figure 1.5). In the ER, disulfide bonds form and undergo isomerization [18]. Of note, the addition of high-mannose glycans begins in the ER (Figure 2.1*)*. The SP is then cleaved before transport of the maturing peptide from the ER to the Golgi where further folding and post-translational processing, e.g. tyrosine sulfation and complex glycan modification, occur [19]. These post-translational modifications (PTMs), particularly glycosylation, have been shown to greatly influence the structuring of mature proteins as well as protein function [20-22].

HIV Env SPs show a high degree of sequence diversity (Figure 2.1*)*; however, several conserved structural features have been identified. Much like the SP of all secreted or membrane proteins, the Env SP includes a positively charged n-region, a hydrophobic core (h-region) and a cleavage domain. These regions strongly influence the rate of processing of Env as it transits through the ER and Golgi [23-26]. This is believed to alter Env synthesis including the trafficking of nascent Env to the ER, the rate of SP cleavage

inside the ER and/or the retention time of Env in the ER. *Asmal et al* concluded that the SP signature at position 12 provides transmitting HIV isolates with higher overall Env expression, which translates into higher Env incorporation into virions [11].

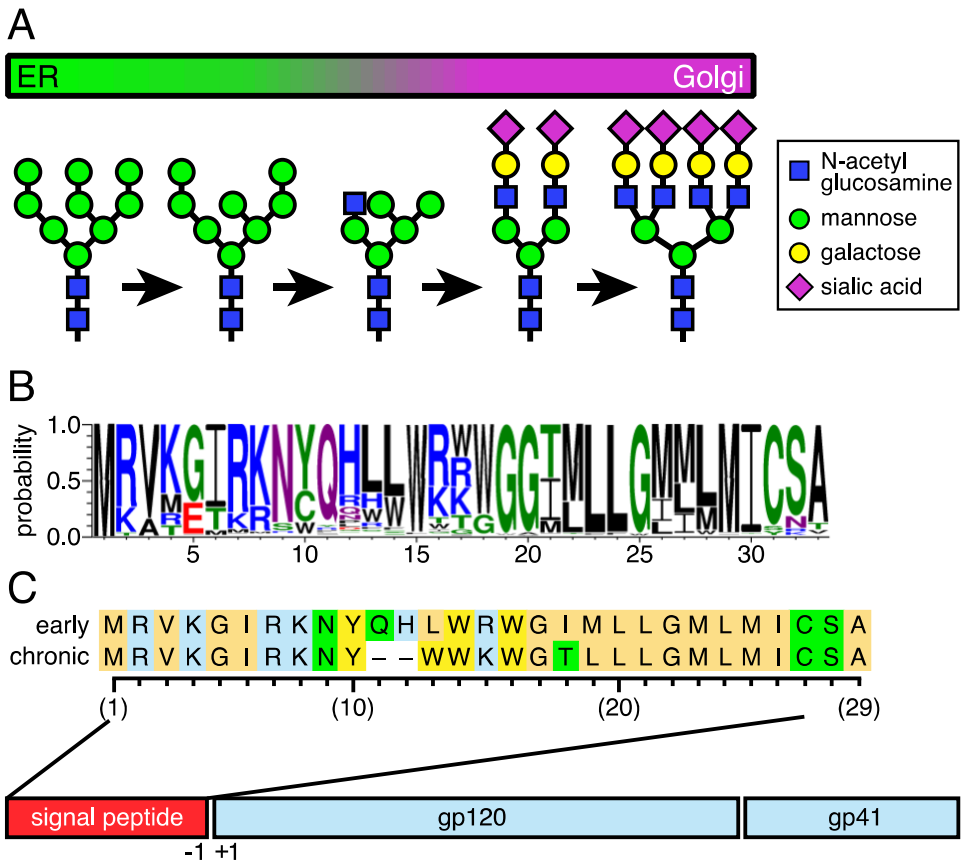


Figure 2.1. HIV Env SP role in early HIV Env processing and glycosylation. (A) N-linked glycans are added in the ER to the asparagine in an N-linked glycosylation sequon of nascent peptides as $Glc_3Man_9GlcNAc_2$. The three Glcs are sequentially removed followed by removal of one Man residue. This glycosidase activity is intimately associated with glycoprotein folding assisted by ER resident chaperones and determines whether a protein traffics to the Golgi or is degraded by the proteasome. Once in the Golgi, the glycans are further modified with the addition of GlcNAc, Gal, Fuc, sialic acids, or other complex saccharide linkages. Gal, galactose; Glc, glucose; GlcNAc, N-acetyl glucosamine; Fuc, fucose; Man, mannose. (B) Sequence logo of natural variation found in 2,025 Clade B HIV Env SP sequences from the Los Alamos National Laboratory HIV Database using Weblogo 3. The height of each symbol indicates relative frequency of each amino acid at that position. Amino acid symbols are color coded for hydrophobicity: blue, hydrophilic; green, neutral; black, hydrophobic. (C) SP amino acid sequence alignment of the SP of the early-replicating isolate bearing the SP transmission signature and of the isolate that does not bear the SP transmission signature. Amino acid residues are color coded for side chain chemistry: orange, nonpolar; blue, basic; green, polar; yellow, aromatic; red, acidic.

There are no studies to date that investigate how natural variation in the SP impacts the overall structure or antigenicity of Env or how SP variation impacts neutralization by monoclonal antibodies (mAbs). Identification of a transmission signature located in a region of the SP sequence that is absent in the mature full-length protein (Figure 2.1), suggests a potential role for SP variation in impacting Env structure and function. Thus, SP variation may be subject to immune pressure despite the fact that this domain is absent from the mature protein.

Given that we now have evidence for SP selection in the context of HIV transmission, we sought to determine whether the natural variation that occurs in SPs impacts Env glycosylation and structure, which in turn could influence Env antigenicity and its ability to be neutralized by bnAbs. Here, we provide evidence that naturally occurring variation in SP can impact both Env glycosylation and structure in a way that might potentially impact the efficiency of replication in early-transmitting viruses. We also show that the SP can impact the neutralization profile of replication competent viruses. Our findings provide evidence that natural variation in the SP of Env can influence a key antigenic feature of gp120 in a major way. In addition, we demonstrate that antigenic manipulation of Env in the context of a vaccine can be carried out by simple modification of the SP.

2.3 Experimental Procedures and Methods

2.3.1 Recombinant gp120 Production and Purification

Two HIV-1 clone sequences from HIV-1-positive patients deposited into the HIV Database at Los Alamos National Library were used in this study: an early-replicating

isolate bearing the SP transmission signature termed 'early-replicating' (Genbank accession #EU289198), and a non-SP transmission signature bearing isolate termed 'chronic' (Genbank accession #EU575870). Both full-length gp120 DNA sequences were synthesized for expression in mammalian cells (ATUM). The constructs were designed such that swapping of the wildtype SP with an alternative SP could be achieved by single digest and insertion of the complimentary SP without alterations to the mature gp120 protein sequence. Using this approach, two additional recombinant gp120 constructs were generated: early-replicating gp120 with chronic SP and chronic gp120 with early-replicating SP. All recombinant gp120s were produced and purified in the same manner. Vectors were transiently transfected into CHO-S-Freestyle cells (Invitrogen) using FreeStyle MAX Reagent and Polyfect (Qiagen). Clonal cell lines were established and subsequently seeded into hollow-fiber cartridges (30 kD MW cutoff) (Fibercell Systems). Protein containing supernatants were harvested daily from the extra-capillary space. Pooled supernatants were then passed over a *Galanthus nivalis* lectin column (Vector Labs) and eluted with 500mM α-methyl-manno pyranoside (Sigma), desalted and passed through a cobalt-chelating column to remove contaminants. Protein was then passed over a superdex-200 26/60 gel-filtration column (GE Healthcare Bio-Sciences). Peak fractions were collected and concentrated with a stirred cell concentrator (Millipore) and dialyzed exhaustively against HEPES, pH 7.4, 150 mM NaCl. Proteins were quantified by UV adsorption at O.D λ_{280} with an extinction coefficient of 1.1 and the values were confirmed by a bicinchoninic acid protein assay (Pierce).

2.3.2 Surface Plasmon Resonance Analysis

Surface plasmon resonance analysis was performed on a Biacore 3000 instrument (GE Life Sciences) using CM5 sensor chips. The data were evaluated with BIAevaluation 4.1 software (GE Life Sciences). The chip surface was activated by injecting 35 µl of a 1/1 mixture of 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide at 5 µl/min. Each of the four gp120s (5 µg/ml in 10 mM NaOAc (pH 5)) were immobilized to a flow cell to a density of approximately 750 resonance units (RU) and blocked with 35 µl of 1 M Tris-HCl (pH 8.0). For a background control, human IgG was immobilized to one flow cell. HBS (pH 7.4), 0.005% Tween p20 was used as running buffer. To evaluate gp120 characteristics Narcissus pseudonarcissus (Daffodil) lectin (200 mM), Ricin Comunis I (Castor Bean) lectin (200 mM), mAb 2G12 (100 nM), soluble tetrameric DC-SIGN (200 mM) mAb 17b (100 mM), and mAb A32 (100 mM) were individually injected over the surface-bound gp120s at a flow rate of 25 µl/min for 300 seconds. After probe injection, the surface was washed for 2 min with running buffer to follow the dissociation from the gp120s. The surfaces were regenerated by injection of 4.5 M $MgCl_2$ at a flow rate of 50 µl/min.

2.3.3 Infectious Molecular Clone Production

The NL4.3 viral backbone construct that has been described previously [27] was used to generate infectious molecular clones (IMCs). We took advantage of the backbone's multiple restriction sites to insert an Env fragment containing the SP (Env position 1-30), the gp120 (position 31-509) and much of the gp41 (position 510-750 out of 854) from isolate SF162 (Accession #EU123924). We also inserted an Nhe1 restriction site into the

CSA SP cleavage motif. Therefore, by simple digest using the endogenous Sac1 restriction site just upstream of the SP and the inserted Nhe1 site in the new NL4.3-SF162env construct (+30), swapping of the SF162 SP for other heterologous HIV Env SPs could be accomplished. A total of 11 isolate sequences were used to generate NL4.3-SF162 SP chimera constructs: (Figure 2.6). All Env and SP cloning fragments were commercially synthesized (ATUM). Sanger sequencing of the wildtype and SP chimera virus constructs was done to confirm cloning (Genewiz).

Viral constructs were transiently transfected into HEK293T using polyfect reagent following the manufacturer's protocol (Qiagen). Supernatants were harvested at day 7 and analyzed for virus production by p24 ELISA (Perkin Elmer). HEK293T supernatants containing virus were then used to inoculate OKT3, IL-2 and retinoic acid activated PBMC cultures. All NL4.3-SF162 293T viral supernatants were passaged through the same PBMC donor's cells to negate donor-to-donor differences. PBMC culture supernatants were harvested on day 7 and analyzed for virus production by p24 ELISA (Perkin Elmer). Aliquoted stocks were frozen at -80 ºC until use.

2.3.4. Infectious Molecular Clone Neutralization Assay

Virus neutralization of IMCs by bnAbs was measured with TZM.bl target cells using a β-galactosidase-based assay [28, 29]. Serial dilutions of mAbs were added to virus in a 96-well plate in duplicate or triplicate and incubated for 24 h at 37 ºC. After incubation, TZM.bl cells were added along with DEAE-dextran and incubated for 48 h. Relative luminescence was measured, and percent neutralization was calculated by comparing mAb-virus incubation to virus alone condition.

2.4 Results

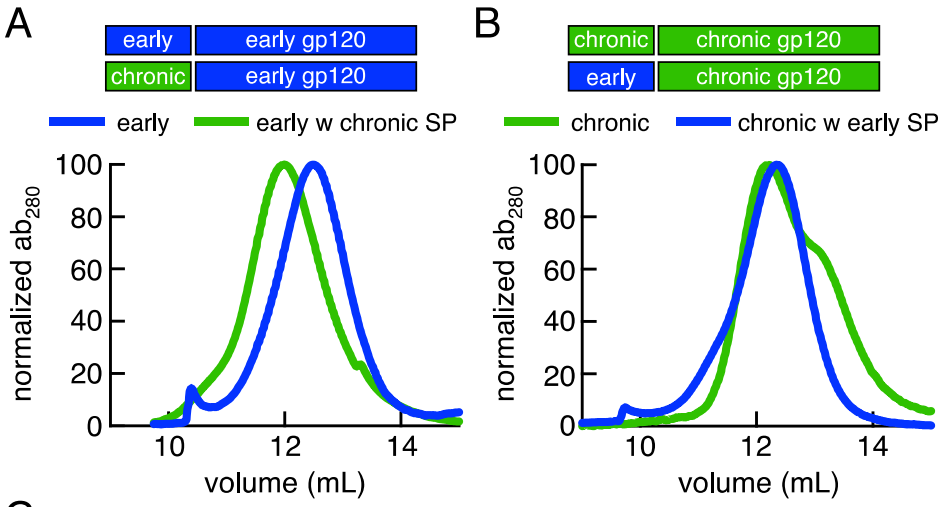
2.4.1 The HIV Env signal peptide influences the molecular weight of gp120.

To address the influence of the SP signature on Env characteristics associated with transmission, recombinant gp120 proteins were generated from viral sequences of HIV-positive patients. We chose two gp120s from isolates previously evaluated in the initial *Asmal et al* report describing the SP signature associated with transmission [11]. First, a subtype B gp120 derived from a viral sequence isolated from an early-replicating isolate was generated. This gp120 contains a basic amino acid histidine at position 12 of the Env SP and is therefore referred to as "early-replicating" (Figure 2.2). A second recombinant gp120 was generated from an isolate from an infected patient that lacked this SP signature. This isolate was termed "chronic" since it did not bear the SP transmission signature (Figure 2.2). Two chimeric gp120s were then generated from these two constructs in which the SPs of the two isolates were swapped, resulting in the early-replicating gp120 with the chronic SP and a chronic gp120 with the early-replicating SP. Using the parental and chimeric gp120 sequences, we sought to determine if their SPs could influence important antigenic features of the gp120s.

Size exclusion chromatography (SEC) provides an approximation of the Stokes radius of a protein. Although SEC does not provide a precise measure of molecular weight (M.W.), it can identify relative differences in the size of structurally related proteins. Using SEC, we asked whether the early-replicating and chronic gp120s encoding their wildtype SPs along with the two proteins encoding swapped SPs exhibited altered M.Ws. The calculated M.W. for the early-replicating gp120 was 102 kDa (Figure 2.2). Despite

containing the identical mature peptide sequence, the early-replicating gp120 with the chronic SP showed a M.W. of 179 kDa (Figure 2.2A). Thus, replacing the early-replicating SP with the chronic SP substantially increased the M.W. of gp120.

Conversely, the chronic gp120 with the early-replicating SP showed a decrease in M.W. compared to the chronic gp120 with its wildtype chronic SP (147 kDa vs 123 kDa) (Figure 2.2B). These SEC data indicate that variations in the SP of Env can have a significant impact on the M.W. of mature gp120 peptides.



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signal peptide	mature gp120	calculated M.W. (kDa)	change (kDa)
early	early	102	
chronic	early	179	↑ 77
chronic	chronic	147	
early	chronic	123	↓ 24

Figure 2.2. HIV Env SP impacts recombinant gp120 molecular mass. Size exclusion chromatography (SEC) chromatograms of the recombinant (A) early-replicating gp120 with its wild-type SP (blue), the chronic SP (green), (B) the chronic gp120 with its wild-type SP (green), and the early-replicating SP (blue) produced in CHO-S cells and purified via *Galanthus nivalis* lectin column. (C) Tabulated molecular mass and molecular mass changes for all four proteins.

2.4.2 The HIV Env signal peptide influences gp120 glycosylation.

The SEC results described above suggested that variations in the SP of Env may influence the maturation process of gp120. Of the PTMs of the Env, glycosylation has been shown to account for a significant fraction of the M.W. of the protein [8]. Therefore, we sought to determine whether the changes in the gp120 M.W. that we observed were due, at least in part, to altered glycan processing.

High-mannose carbohydrates are the initial glycans added to nascent peptides (Figure 2.1A) and are characteristic of immature glycoproteins. They are also associated with mature proteins that transit through the ER and Golgi in an accelerated way. Conversely, complex carbohydrates exhibit a higher M.W. than high-mannose glycans (2.4 kDa vs 1.2 kDa) and are not formed until later in the processing pathway. With an average of 25 PNGSs on each gp120, changing all 25 from high-mannose occupied PNGSs to all complex glycan occupied PNGs would result in a ~30 kDa shift in M.W.

To quantitate the relative amount of high-mannose glycans on the gp120s, we employed the *Narcissus pseudonarcissus* lectin, which is specific for α-linked mannose residues (Man), [30] in a surface plasmon resonance (SPR) assay. The early-replicating and chronic Env proteins, along with the two SP chimeras were immobilized to the surface of a biosensor chip. *Narcissus pseudonarcissus* lectin was passed over each of the four chip surfaces and the total accumulation of lectin bound to each surface was measured over 300 seconds.

This demonstrated significant Man content on the early-replicating gp120 with the wildtype SP (178.0 RU) (Figure 2.3A, Left). Man content was shown to be decreased when

we probed the early-replicating gp120 with the chronic SP (130.2 RU) (Figure 2.3A, Left). The inverse was found when looking at the two chronic gp120s; swapping the SP of the chronic gp120 to that of the early-replicating SP caused an increase in Man content (159.5 RU vs 225.0 RU) (Figure 2.3A, Right). These results indicate that the SP alone can significantly influence the Man content of gp120. More specifically, the early-replicating SP, which may favor more rapid transit through the ER and Golgi compartments, increases the relative amount of high mannose carbohydrate present on the two gp120s we evaluated.

We then probed each of the four gp120s with a lectin derived from *Ricinus communis* that preferentially binds complex oligosaccharides ending in galactose (Gal) [31]. The early-replicating gp120 showed little detectable reactivity to *Ricinus communis* lectin (17.8 RU) (Figure 2.3B, Left). In contrast, the same gp120 encoding the chronic SP reacted strongly (494.4 RU) to *Ricinus communis* lectin. Thus, the chronic SP substantially increased the amount of complex oligosaccharides ending in Gal in the early-replicating gp120.

Consistent with this observation, the chronic gp120 encoding its wildtype SP also reacted strongly with *Ricinus communis* lectin (558.8 RU), while the same protein encoding the early-replicating SP showed little or no reactivity with *Ricinus communis* lectin (12.6 RU) (Figure 2.3B, Right).

Taken together, these results indicate that the chronic SP promotes the addition of complex glycans bearing terminal Gal.

Overall, the manner in which the two SPs differentially impacted the presentation of mannose and complex carbohydrate was consistent insofar as the early-replicating SP

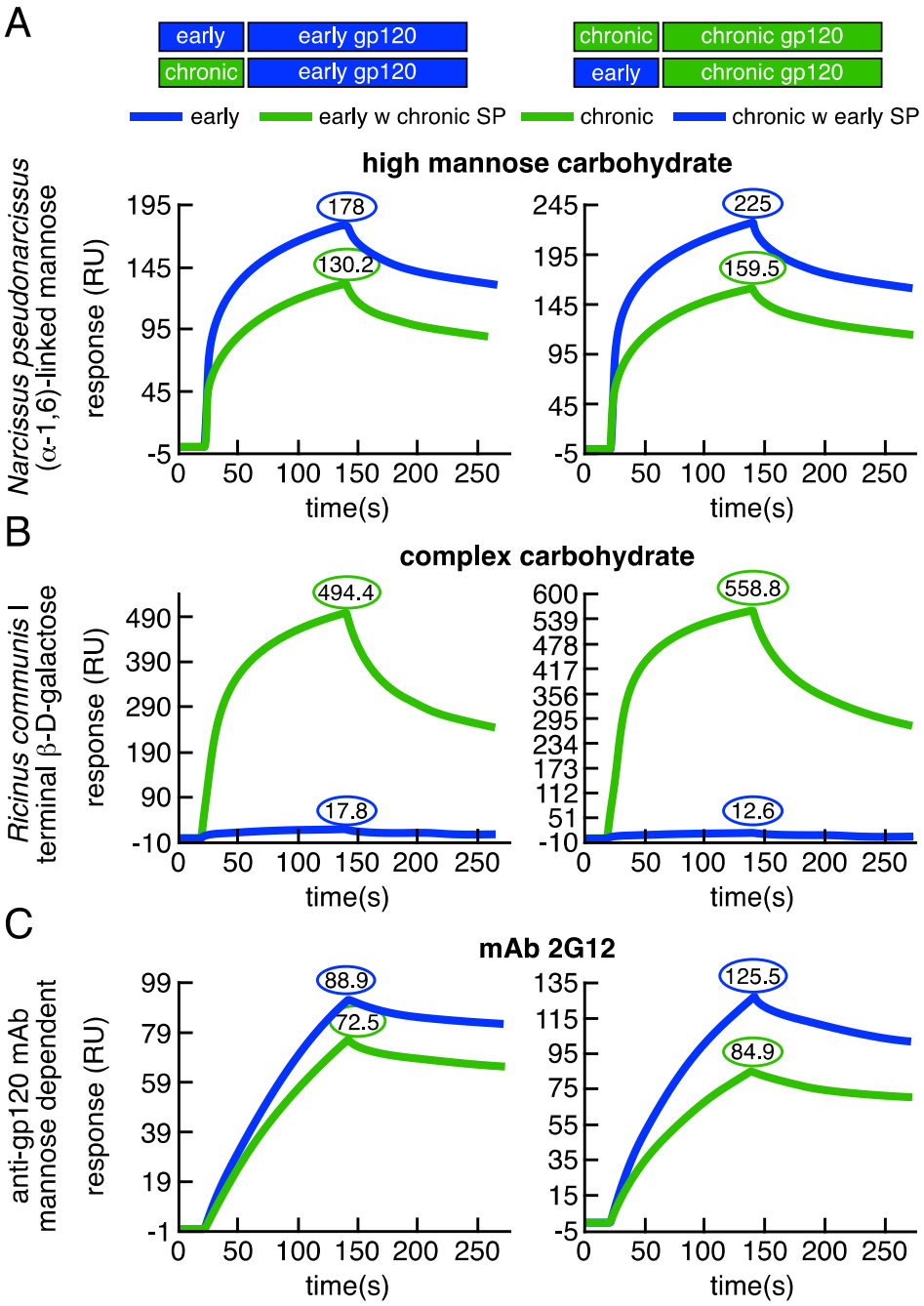

Figure 2.3. HIV Env SP influences gp120 glycosylation profile. Four recombinant gp120s were evaluated for glycan composition in a surface plasmon resonance (SPR) assay probing with (*A*) *N. pseudonarcissus* lectin specific for α-linked mannose residues and (*B*) *R. communis* I lectin specific for oligosaccharides ending in galactose. (*C*) The four gp120 were also probed with the glycan-dependent mAb 2G12. (*Left*) Sensograms of the early-replicating gp120 with wild-type SP (blue) and with the chronic SP (green), and (*Right*) sensograms of the chronic gp120 with its wild-type SP (green) and with the early-replicating SP (blue). Peak response units (RU) are presented and circled above each curve.

appeared to favor the presentation of high-mannose on either the early-replicating or the chronic protein while the chronic SP favored at least one form of complex carbohydrate.

HIV gp120 monoclonal antibodies (mAbs) specific in whole or in part to glycan patches on gp120 have been isolated from HIV infected individuals [32-34]. A number of these mAbs show broad and potent neutralizing activity [32, 34, 35]. 2G12 is the prototypical glycan-dependent mAb [33, 36]. It recognizes a discontinuous di-mannose epitope located in the C3 region and the base of the V3 loop in an area often referred to as the silent face of gp120 [36]. When probed with mAb 2G12, the early-replicating gp120 encoding the chronic SP showed decreased reactivity compared to the same gp120 encoding the early-replicating SP (72.5 RU vs 88.9 RU) (Figure 2.3C, Left). The chronic gp120 with the early-replicating SP showed increased binding to 2G12 compared to the chronic gp120 with its wildtype chronic SP (125.5 RU vs 84.9 RU) (Figure 2.3C, Right).

Taken together, these results demonstrate that the reactivity of glycan specific antibodies to gp120 can be influenced by the SP. Regardless of mature gp120 sequence, the SP of the early-replicating Env biased the mature protein to a high Man, low complex carbohydrate profile. Conversely, the SP of the chronic gp120 biased the gp120 to an increased complex carbohydrate profile. Because Man is roughly half the molecular weight of an average complex glycan (~1.2 versus ~2.4 kD), and carbohydrate comprises about half the mass of a gp120 surface protein, these alterations in glycan processing are likely playing a major role in the M.W. shift observed for these peptides shown in Figure 2.2.

2.4.3 The HIV Env signal peptide influences gp120: DC-SIGN interaction.

C-type lectin receptors are thought to facilitate mucosal transmission of HIV [37, 38]. Among these receptors, DC-SIGN is perhaps the most extensively characterized [39-41]. The carbohydrate-recognition domain of DC-SIGN reacts with high affinity to the high-mannose residues that decorate gp120 [42-44]. Considering the capacity of the SP to influence the glycan content of gp120, we sought to determine whether SP variation could also influence gp120 interactions with DC-SIGN.

Using tetrameric recombinant soluble DC-SIGN as a probe, we determined that the early-replicating gp120 with its wildtype SP showed higher reactivity to DC-SIGN than the early-replicating gp120 encoding the chronic SP (208.2 RU vs 130.6 RU) (Figure 2.4A). When the wildtype SP of the chronic gp120 was replaced with the early-replicating SP, DC-SIGN reactivity nearly doubled (184.5 RU vs 308.4 RU) (Figure 2.4B).

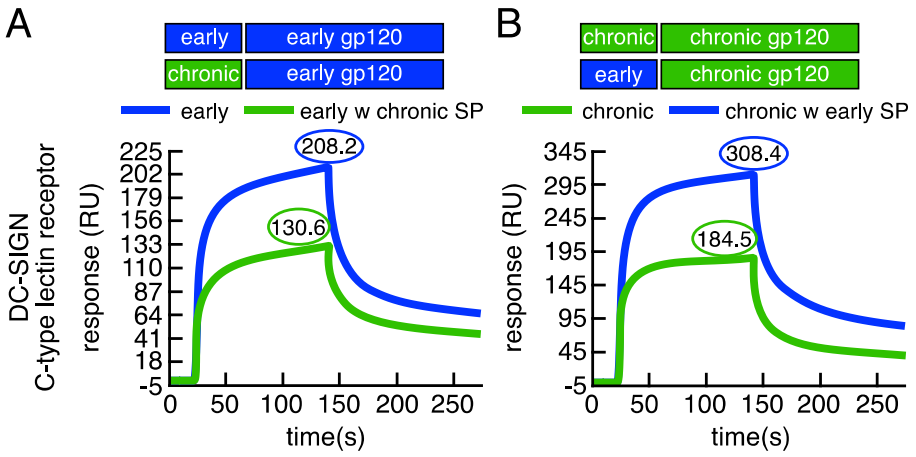

Figure 2.4. HIV Env SP influences DC-SIGN reactivity to gp120. (A) The early-replicating gp120 with its wild-type SP (blue) and with the chronic SP (green), and (B) the chronic gp120 with its wild-type SP (green) and the chronic gp120 with the early-replicating SP (blue) were evaluated for reactivity to soluble tetrameric DC-SIGN, C-type lectin receptor expressed on dendritic cells, in an SPR assay. Peak response units (RU) are presented and circled above each curve.

These results are consistent with the results presented above indicating that the early SP increases the relative proportion of Man presented on gp120, and suggest that the sequence variation encoded in the SP can alter interactions with lectin receptors, which may be relevant in the context of mucosal transmission.

2.4.4 The HIV Env signal peptide influences gp120 antigenicity.

Altered glycosylation profiles of Env can greatly influence structural and antigenic features of gp120 [45]. A prominent feature of HIV gp120s involves the induction of buried epitopes following CD4 ligation (CD4i epitopes). Such changes are conserved in both HIV-1 and HIV-2 and have a significant impact on antigenicity and susceptibility of viruses to neutralizing antibodies [46, 47]. To understand if the changes in gp120 described above can influence protein antigenicity, we measured reactivity with structure-specific gp120 mAbs. mAbs 17b and A32 recognize CD4i epitopes [48-51].

As a control for this measure, and to establish that our SPR–based assay was able to detect CD4i epitopes that are impacted by glycosylation, we employed a wildtype subtype A gp120, 92UG037, and the same gp120 with a PNGS disrupting mutation, N144Q. These proteins were probed with mAbs 17b and A32. In the absence of CD4, mAb 17b reacted well to the wildtype 92UG037 gp120 (22.9 RU) (Figure 2.5A, Left). This reactivity was completely abrogated in the N144Q PNGS mutant gp120 (0.0 RU) (Figure 2.5A, Left). The same trend was observed when probing these two gp120s with the mAb A32, in that the wildtype gp120 had significant mAb A32 reactivity while the N144Q PNGS mutant had greatly reduced mAb A32 reactivity (54.6 RU vs 10.7 RU) (Figure 2.5A,

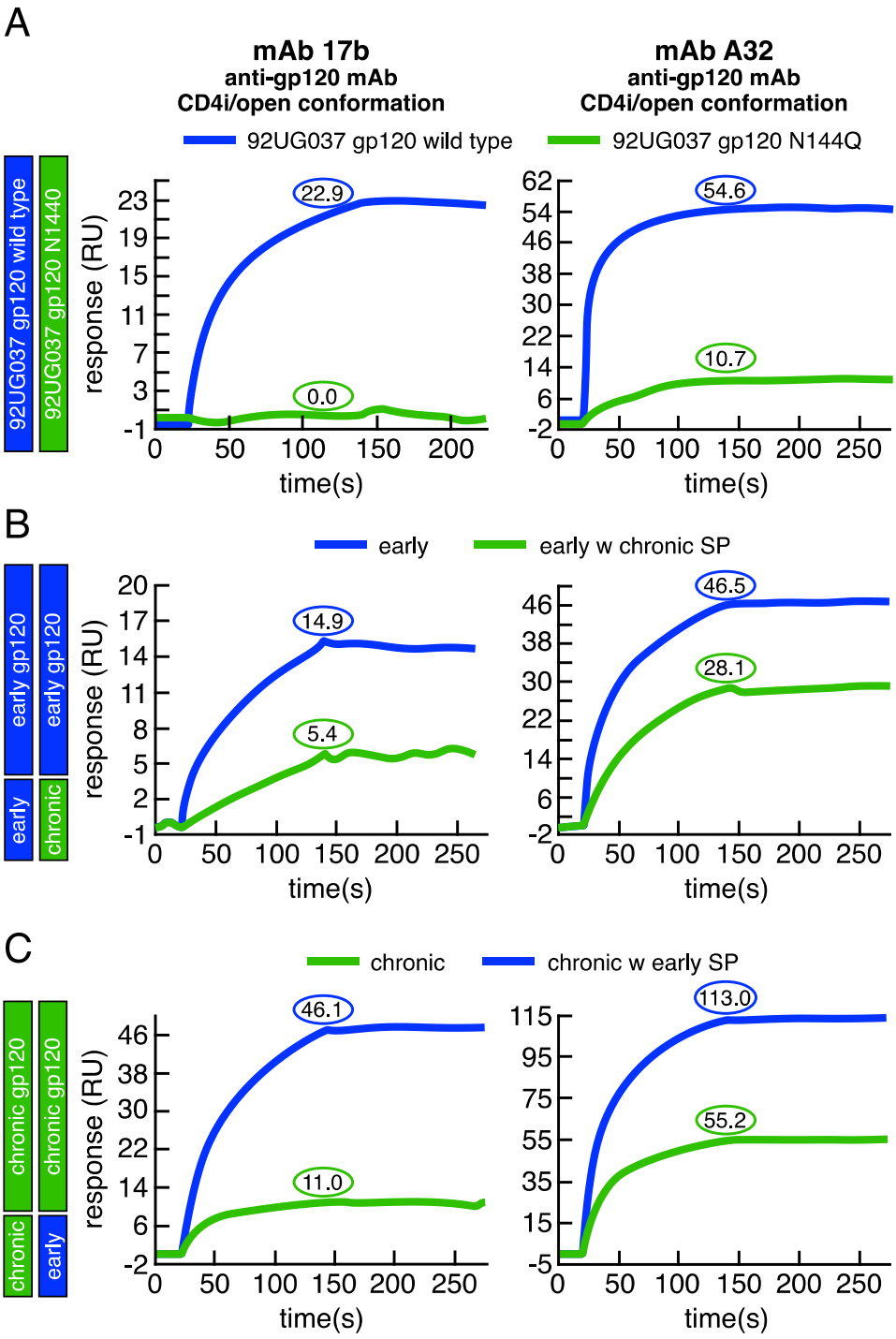

Figure 2.5. HIV Env signal peptide influences gp120 structure and antigenicity. (A) Subtype A gp120 92UG037 (blue) and the PNG mutant 92UG037 N144Q (green) were probed for reactivity with the CD4i mAbs 17b (Left) and A32 (Right) in an SPR assay to determine the impact of glycosylation on gp120 antigenicity. (B) Early-replicating gp120 with wild-type SP (blue) and early- replicating gp120 with the chronic SP (green) were probed with mAbs 17b (Left) and A32 (Right) in an SPR assay. (C) Chronic gp120 with chronic SP (green) and chronic gp120 with the early-

replicating SP (blue) were also probed with the mAbs 17b (Left) and A32 (Right). Peak response units (RU) are presented and circled above each curve.

Right). These data confirm that altered glycosylation influences the ability of gp120 to adopt the CD4i open conformation recognized by mAbs 17b and A32.

We next determined whether sequence variation in Env SPs could influence the antigenicity of gp120. To test this hypothesis, we probed our four recombinant gp120s for reactivity with mAbs 17b and A32 in the absence of CD4. The reactivity to mAb 17b was reduced by more than half when the SP of the early-replicating gp120 was swapped from its wildtype SP to that of the chronic SP (14.9 RU vs 5.4 RU) (Figure 2.5B, Left). Additionally, the same trend of reduced reactivity of the early-replicating gp120 with the chronic SP was observed when the two proteins were probed with mAb A32 (46.5 RU vs 28.1 RU) (Figure 2.5B, Right).

We then probed the chronic gp120 with its wildtype SP versus the early-replicating SP with mAbs 17b and A32. We observed low reactivity of mAb 17b with the chronic gp120 with its wildtype SP (11.0 RU) (Figure 2.5C, Left). However, reactivity to mAb 17b increased four-fold when the SP of the chronic gp120 was swapped to that of the early-replicating SP (46.1 RU) (Figure 2.5C, Left). When the two chronic gp120s were probed with mAb A32, reactivity of the mAb to the chronic gp120 with the early-replicating SP was increased two-fold when compared to the chronic gp120 with its wildtype SP (55.2 RU vs 113.0 RU) (Figure 2.5C, Right).

Taken together, these results show that the SP of Env can influence the reactivity of CD4i mAbs to mature gp120s. These data suggest that the glycosylation differences induced by natural SP variation can greatly influence the antigenicity of gp120.

2.4.5 The HIV Env signal peptide impacts viral neutralization

The above experiments sought to characterize the impact of the HIV Env SP using recombinant peptides. No studies to date have investigated the impact of natural variation in the Env SP on replication competent infectious molecular clones (IMCs). To investigate the impact of SP variation on viral neutralization we generated infectious molecular clones using an NL4.3 isolate backbone construct that contains the entire HIV genome and produces replication competent IMCs when transfected into mammalian cell cultures. Modification of the viral backbone was done by simple cloning digest to replace the SP, the gp120 and most of the gp41 of the NL4.3 construct to that of the well characterized isolate SF162. The new NL4.3-SF162env construct was additionally modified to create chimera SP constructs in that the SP of the SF162 Env was swapped individually with 11 diverse isolate SP sequences (Figure 2.6). The NL4.3-SF162env IMC with the SF162 SP is referred to as wildtype (WT), while the SP chimera IMCs are referred to by their SP isolate name (i.e. NL4.3-SF162env with the SP of 398.F1 isolate is referred to as 398.F1). The resulting constructs were transfected into 293T cells and were passaged once through activated PBMC culture.

Because we hoped to compare the neutralization profiles of these viruses without differences in infectivity as a confounding factor, we first tested whether the SP impacted *in vitro* infectivity by using a TZM.bl β-galactosidase-based infectivity assay. We found that all 12 (wildtype and 11 chimera SP) viruses had similar infectivity profiles in this assay (Figure 2.7). This shows that the SP does not significantly impact the *in vitro* infectivity of TZM.bl target cells by these IMCs. However, we are unable to make conclusions about the infectivity of these IMCs in regard to primary cells or *in vivo*.

A

	isolate name	abbreviation	accession number	subtype	neutralization tier	country of origin	stage at isolation
Env	SF162	SF162	EU123924	B	1	US	late
signal peptide	93MW965_26	MW965	U08455	C	1A	Malawi	late
	HXB2-LAI-IIIB-BRU	HXB2	K03455	B	1	France	late
	398_F1_F6_20	398.F1	HM215312	A1	1	Tanzania	late
	CH119 07_BC	CH119.10	EF117261	CRF_07.BC	2	China	late
	H078_14	H078	EF210733	B	3	Peru	late
	TRJO_58	TRJO	AY835450	B	3	U.S.	early
	CE703010217_B6	CE0217	FJ443575	B	2	Malawi	late
	CNE8	CNE8	HM215427	CRF_01.AE	3	China	late
	TRO.11	TRO.11	AY835445	B	3	Italy	early
	271.11	271.11	EU513197	CRF_02.AG	2	Cameroon	early
	257.31	257.31	EU513185	CRF_02.AG	2	Cameroon	early

B

```
                1         10        20        30        38
SF162:          MRVKGIRKNYQHL-----WRGGT---LLLGMLMICSAS
MW965:          MRVMGIRKNWQQL-----WIWGI---LGFGMLMICSAS
HXB2:           MRV---KEKYQHLWRWG-WRWGT---MLLGMLMICSAS
398.F1:         MRARGIQRNWQQW-----WIWGI---LGFWTVMICSAS
CH119.10:       MRVTGIRKNYRHL-----WRWGT---MLLGMLMICSAS
H078:           MRVTGIMRNCQHL-----WKWGM---MLLGMLMICSAS
TRJO:           MRVMGIRKNYQHL-----WRWGTMGMMLLGILMICSAS
CE0217:         MRVTGTQRNYPRWWIWGWWIWGI---LGFWMLLNCSAS
CNE8:           MRVKETQMNWPNL-----WKWGT---LILGLVIICSAS
TRO.11:         MRAKGIRKNCQHL-----WIWGT---MLLGMLMICSAS
271.11:         MRVMGIQRNYPPL-----WRWGT---MIFWIMTMCSAS
257.31:         MRVRGIQRNCPPL-----WRWGI---IIFWMLIICSAS
```

Figure 2.6. Sequence information for Env signal peptide chimera IMCs. (**A**) Details for the isolates from which the signal peptide (SP) sequence were derived for the generation of SP chimera IMCs. "Stage at isolation" refers to the timing of isolate isolation based on estimated or documented date of infection: early (<6 mo after infection) or late (>6 mo after infection). (**B**) Amino acid sequence alignment of the SPs used in the generation of the SP chimera IMCs. Alignment was generated using MAFT alignment program via MegAlign Pro software (Lasergene).

To investigate the impact of SP variation on virus neutralization profile we employed the same infectivity assay as described above with an added 24 h pre-incubation of anti-gp120 mAb with the IMCs. Because we show that the SP can impact the accessibility of neutralizing epitopes on gp120, we reasoned that the SP may influence the viral neutralization by mAbs targeting the V2 loop of gp120, which is a major target of broadly neutralizing antibodies (bnAbs). We therefore tested these SP chimera IMCs for

neutralization sensitivity to anti-V2 loop neutralizing mAbs. Many of the well-characterized V2-targeting bnAbs target, in part, conserved glycans in this region [52].

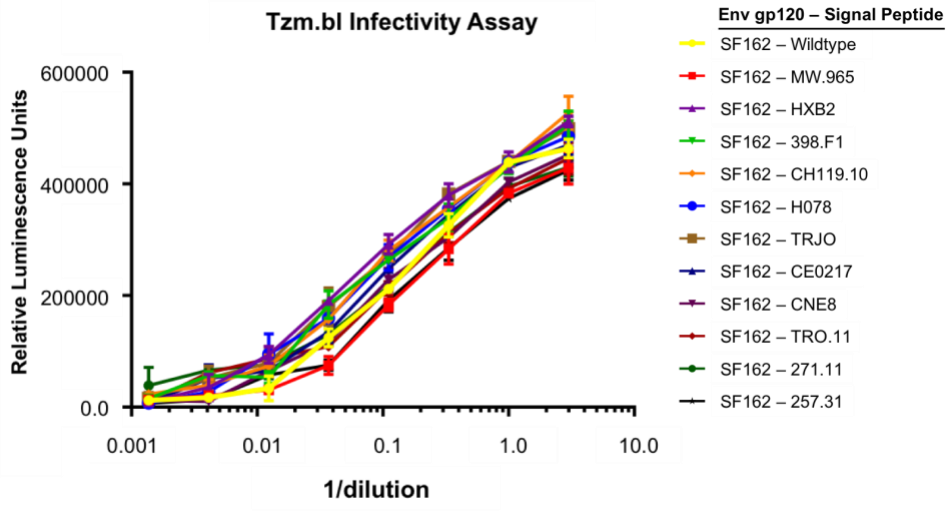

Figure 2.7. Infectivity of Env signal peptide chimera IMCs. Viral infectivity was determined by serially diluted amounts of chimera NL4.3-SF162envSP virus incubated with TZM.bl target cells. Infectivity was measured 48h later by β-galactosidase activity assay.

The chimera IMCs were first tested for neutralization by CD4-Ig, a known strong inhibitor of isolate SF162 infectivity in this assay. All chimera IMCs were comparably neutralized by CD4-Ig with ≥50% neutralization at 0.02 ug/mL (Figure 2.8). This suggests that these SP chimera IMCs are not differentially impacted by CD4 binding in a detectable way. We also tested neutralization by the irrelevant anti-parvovirus B19 mAb 1418. Neutralization by mAb 1418 was similar between our panel of SP chimera IMCs. This control data shows the extremes in neutralization sensitivity and susceptibility for our panel of SP chimera IMCs.

To investigate the impact of SP variation on viral neutralization by anti-V2 mAbs, we tested the neutralization of these chimera IMCs by the anti-V2 mAbs 2158, 830A and 697-D (Figure 2.8). The WT IMC was largely resistant to neutralization by the anti-V2 mAb 2158, only reaching above 50% neutralization at the highest concentration tested (100 μg/mL). Interestingly, some but not all of the SP chimera IMCs were susceptible to neutralization by mAb 2158 (Figure 2.8). The 398.F1 IMC, reached ~75% neutralization at 20 μg/mL and ~90% at 100 μg/mL. This suggests that natural sequence variation in the SP can alter the V2 domain of gp120 of an IMC in a way that makes the virus more susceptible to neutralization by the mAb 2158.

While we found that some SP variants can increase the susceptibility to neutralization by the V2 bnAb 2158, this was not always the case. When we investigated the neutralization profile of these SP chimera IMCs by another anti-V2 mAb, 830A, we found that the neutralization profile was not significantly altered (Figure 2.8). For the SP chimera IMCs that we tested, we found that all were largely susceptible to neutralization by mAb 830A (i.e. ~50% at 4 μg/mL).

We hypothesized that in our investigation of viral neutralization by anti-V2 mAbs it would be likely to find no change or increased susceptibility to mAb neutralization and unlikely to find a SP chimera IMC that was more resistant to neutralization by mAbs. Surprisingly, we found that the WT IMC was highly susceptible to neutralization by mAb 697-D (~45% neutralization at 4 μg/mL and ~75% at 100 μg/mL), while the 398.F1 IMC didn't reach above 20% neutralization by mAb 697-D at the highest concentration tested (100 μg/mL) (Figure 2.8). This suggests that natural SP variation can impact antigenic characteristics of Env associated with neutralization by anti-V2 mAbs.

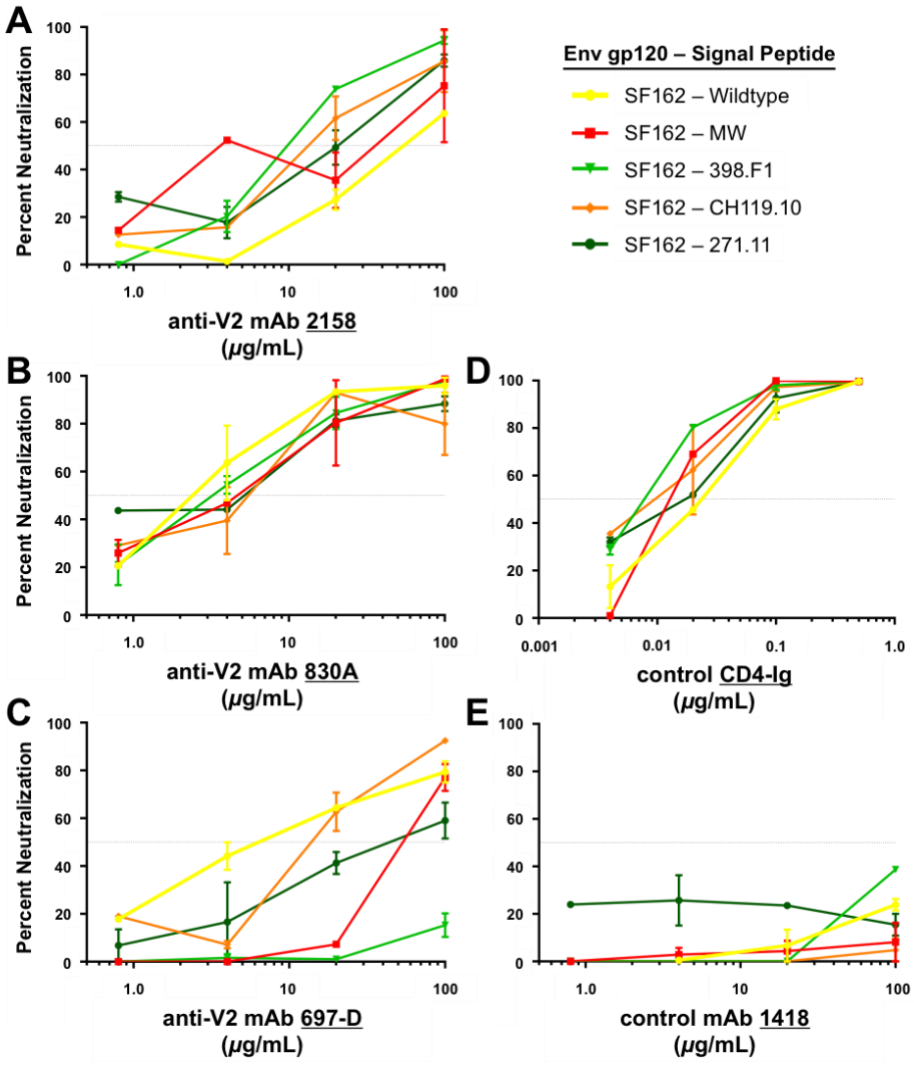

Figure 2.8. HIV Env signal peptide influences IMC neutralization by some anti-V2 domain mAbs. Viral neutralization was determined by chimera NL4.3-SF162envSP virus incubation with serial diluted the anti-V2 mAbs, as well as positive and negative controls for 24h prior to the addition of TZM.bl target cells. Infectivity was measured 48h later by β-galactosidase activity assay. Preincubation was done with (**A**) anti-V2 mAb 2158, (**B**) anti-V2 mAb 830A, (**C**) anti-V2 mAb 697-D, (**D**) control CD4-Ig (positive control) and (**C**) irrelevant parvovirus B19 mAb 1418 (negative control). Percent neutralization was calculated by comparing infectivity of mAb/control preincubation and no preincubation conditions for each virus.

Taken together this data shows that the SP alone can impact the neutralization profile of IMCs. While no association between the isolate from which the SP was derived

and the neutralization profile by anti-V2 mAbs could be identified, we clearly show that the natural variation found in some SPs can greatly impact the susceptibility of these IMCs to neutralization by these V2 mAbs. We show that natural variation in the SP can increase susceptibility of IMCs to some anti-V2 mAb neutralization. We also show that some SP variations can also significantly increase resistance to neutralization by V2 mAbs, a remarkable finding considering that the SP is not present in the mature Env that coats these IMCs.

2.5 Discussion

The HIV-1 Env is heavily glycosylated [8, 53], and alterations to key PNGSs can influence its structure, function, and susceptibility to neutralizing antibodies [22, 36, 54]. Evidence of a connection between Env glycosylation and HIV transmission has also been described [5]. Previous studies have demonstrated that early-replicating Envs tend to encode fewer PNGSs and shorter variable regions in which many PNGSs are located [12, 13]. Additionally, the glycans that decorate the PNGSs of early-replicating Env contain greater high-mannose content compared to Envs isolated from chronic replicating viruses [14]. Recently, a second signature of transmission has been described that involves the Env SP of early-replicating viral isolates [11, 16]. This SP transmission signature has also been described in SIV and SHIV transmission studies [17]. The identification of this signature suggests that the SP may influence the transmission bottleneck that is established around the time of transmission. Understanding the effect of natural variation in the SP coding sequence on the Env protein structure and antigenicity is important insofar as Env is the major target for neutralizing antibodies. In this report, we show that the SP of the HIV Env

protein can impact both the antigenicity of gp120 and the susceptibility to anti-V2 antibodies. This is the first demonstration that natural variation in the SP of Env can impact neutralization susceptibility in IMCs. These findings are remarkable in that the SP of Env is not present in the mature Env protein that decorates mature virions.

The identification of a transmission signature located in the SP of Env by *Asmal* and colleagues [11] implies that the SP is a target of selective pressure during the early events of transmission. Previous studies in which the SP of HIV-1 Envs have been altered or swapped with SPs from unrelated proteins have been shown to alter the rates of Env processing [23-25, 55]. The conclusions from those studies confirm the importance of certain key features of SPs, including the charged n-region. In the present study, we used the natural variation found in subtype B Envs from two independent patient isolates that incorporate the presence or not of the SP transmission signature. We found that the SP can significantly influence the glycan profile and antigenicity of gp120. We also found by production of infectious molecular clones with chimera SPs that natural variation found in SP coding sequences from a range of HIV subtypes can greatly impact the susceptibility to viral neutralization by anti-V2 bnAbs. This suggests that the observed high degree of sequence variation in SPs can influence Env structure and antigenicity in a manner that has not previously been recognized.

Our findings implicate the SP of Env as a potential regulator of PTMs that influence functional characteristics of gp120 associated with transmission. We found that by swapping the SP of Envs in a way that either inserted or removed the SP transmission signature, the molecular weight of the resulting gp120 was significantly altered, predominantly by a modification of the carbohydrate content of gp120. Thus, these data

establish a clear connection between natural variations found in the Env SP and the gp120 carbohydrate profile. It is noteworthy that a domain that does not appear in the mature protein can alter the glycan shield, which plays a key role in immune evasion.

Env has been shown to bind to and interact with multiple host surface proteins and receptors, and affinities for these receptors can change during the course of infection and disease progression [56]. One such receptor, believed to be relevant to HIV transmission is the C-type lectin receptor DC-SIGN, which is expressed on the surface of dendritic cells that survey mucosal tissues [39]. After antigen capture, these DC-SIGN$^+$ dendritic cells migrate to secondary lymphoid tissues and present the captured antigen to T-cells [57]. DC-SIGN captured HIV has been shown to promote trans-infection of CD4$^+$ T-cells through its interaction with viral Env [39]. This capture and presentation of virus particles to target cells is believed to play a role in mucosal transmission of HIV. We show that the interaction dynamics between DC-SIGN and Env can be altered by variations in the Env SP. This result raises the possibility that one of the selective forces associated with variation in the SP may involve modulation of affinity for DC-SIGN and other C-type lectin receptors involved in transmission. The SP of ebolavirus glycoprotein has been implicated as a determinant of DC-SIGN engagement by modulating the incorporation of high-mannose carbohydrates into the glycoprotein [58]. These data also raise the potential for the SP to impact the binding of other receptors relevant to HIV transmission, like integrin $\alpha_4\beta_7$.

Consistent with the effects of glycans on Env structure [45], we find that variations in Env SP can substantially influence the antigenicity of gp120. We probed the two native gp120s along with the two SP hybrids with the conformation specific mAbs 17b and A32

that preferentially recognize gp120s that adopt a CD4i open conformation. We determined that SPs do impact the reactivity of Env with both mAb 17b and A32. The two gp120s with the early-replicating SP reacted more efficiently with these mAbs compared with the two gp120s that lacked the early-replicating SP signature. Of note, Envs that react with mAbs that recognize the CD4i conformation are associated with greater susceptibility to antibody neutralization [59].

To test the susceptibility to antibody neutralization, we generated replication competent chimera IMCs in which the SP had been swapped to naturally occurring isolate SP sequences. Because the V2 domain of gp120 contains conserved PNGSs targeted by neutralizing mAb and its structure is highly susceptible to altered glycosylation, this panel of SP chimera IMCs was subjected to viral neutralization by anti-V2 mAbs. We show that there is a range in the effects of SP variation on antibody neutralization. In some cases, a SP chimera IMC does not alter susceptibility to a particular anti-V2 mAb while increasing susceptibility to another. The finding that our SP chimera IMCs had either unaltered neutralization profiles or had increased susceptibility to neutralization was anticipated. In many cases, generating mutations or chimera proteins promotes instability in the mature protein, leading to greater susceptibility to antibody neutralization. However, we hypothesized that it would be unlikely to generate a SP chimera IMC that would significantly increase the resistance to an anti-V2 mAb. This, however, was the case for one such anti-V2 mAb: some SP chimera IMCs were rendered more resistant to neutralization by mAb 830A. This shows that the SP, which is not present in the virion, can modulate characteristics of Env that influence susceptibility to neutralization by mAbs, including bnAbs. While a connection between characteristics of the isolate from which

each SP is derived and the neutralization profile of the resulting chimera IMC are currently unknown, the proof-of-concept that natural variation in the SP can regulate neutralization sensitivity is powerful.

That HIV has adapted a mechanism that can modulate shielding of immune pressure on a functional protein by chance variation in a non-mature coding sequence is intriguing. It appears likely that HIV has taken advantage of the natural SP variation that arises through random mutations to counter the selection by host immune pressure for or against characteristics that may relate to viral fitness. This mechanism suggests the SP may be used as another tool of the virus in the tug-of-war between the host adaptive immune response and viral escape. As the infection progresses, immune pressure builds, selecting against less shielded variants. This allows increased prevalence of more shielded variants, with presumably less transmission-fitness. Minor variants that maintain less shielded Env, however, provide the viral swarm with the needed transmission-fitness to pass the infection to new recipients.

It is important to note that the identification of a SP transmission signature at position 12 was achieved without any predisposition toward identifying sequences that would alter PTMs or antibody neutralization. Additional studies are required to define the rules by which SP sequences can influence PTMs insofar as SP sequences hold the potential to influence other characteristics of transmission-fitness.

Finally, these findings support the concept that SP variations can be utilized in the production of recombinant proteins to optimize the glycan profile and antigenicity of vaccine immunogens. Much work has been done toward producing optimized vaccine

antigens that will hopefully elicit broadly neutralizing antibodies [60]. The results presented herein provide a new tool that may be useful in that effort.

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Chapter 3:

mAbs TARGETING A HELICAL V1V2 CONFORMATION PRESENT ON INCOMPLETELY PROCESSED HIV ENV gp120 INHIBIT BINDING TO INTEGRIN $\alpha_4\beta_7$

3.1 Abstract

HIV is gut-tropic and the disruption of the GALT during acute/early HIV infection plays an important role in HIV pathogenesis and disease progression. However, the basis for the gut-tropic nature of acute HIV infection is unknown. Sieve analysis of the RV144 HIV vaccine trial, the only HIV vaccine trial with moderate efficacy, found that key residues of the V2 loop of HIV Env gp120, K^{169} and I^{181}, were the target of vaccine-induced immune pressure and associated with reduced risk of HIV acquisition. The integrin $\alpha_4\beta_7$ is a lymphocyte surface receptor that mediates homing to the GALT via its interaction with its natural ligand, MAdCAM. The HIV envelope protein gp120 binds $\alpha_4\beta_7$. The binding sites for $\alpha_4\beta_7$ on the V2 loop of gp120 are a highly conserved LDV/I motif located at positions 179-181 and a cryptic QKE motif at positions 170-172 located 7 residues upstream. These binding sites overlap with the RV144 sieve analysis sites. We report here using cell-surface expressed $\alpha_4\beta_7$ and a soluble $\alpha_4\beta_7$ heterodimer that the affinity for gp120 is similar to that of MAdCAM, and that the gp120 V2 loop can mimic a physiological role of $\alpha_4\beta_7$ in lymphocyte adhesion. We show that $\alpha_4\beta_7$ antagonists (gp120 V2 loop mimetics and anti-$\alpha_4\beta_7$ mAbs) and a specific class of anti-V2 mAbs disrupt this adhesion interaction. The V2 mAbs that inhibit the interaction, CH58, CH59 and CAP228-16H, recognize the V2 loop of gp120 in a helical, coiled conformation. Interestingly, other V2 mAbs that recognize overlapping residues but in the β-barrel conformation found on SOSIP-stabilized Env trimers do not inhibit this interaction. We also show that the CH58 recognized helical, coiled conformation is present on the surface of infected cells and virions but appears to be present on incompletely processed gp120. This data suggests that non-fully processed Env presents the V2 in a helical conformation that exposes the $\alpha_4\beta_7$ binding site(s) potentially

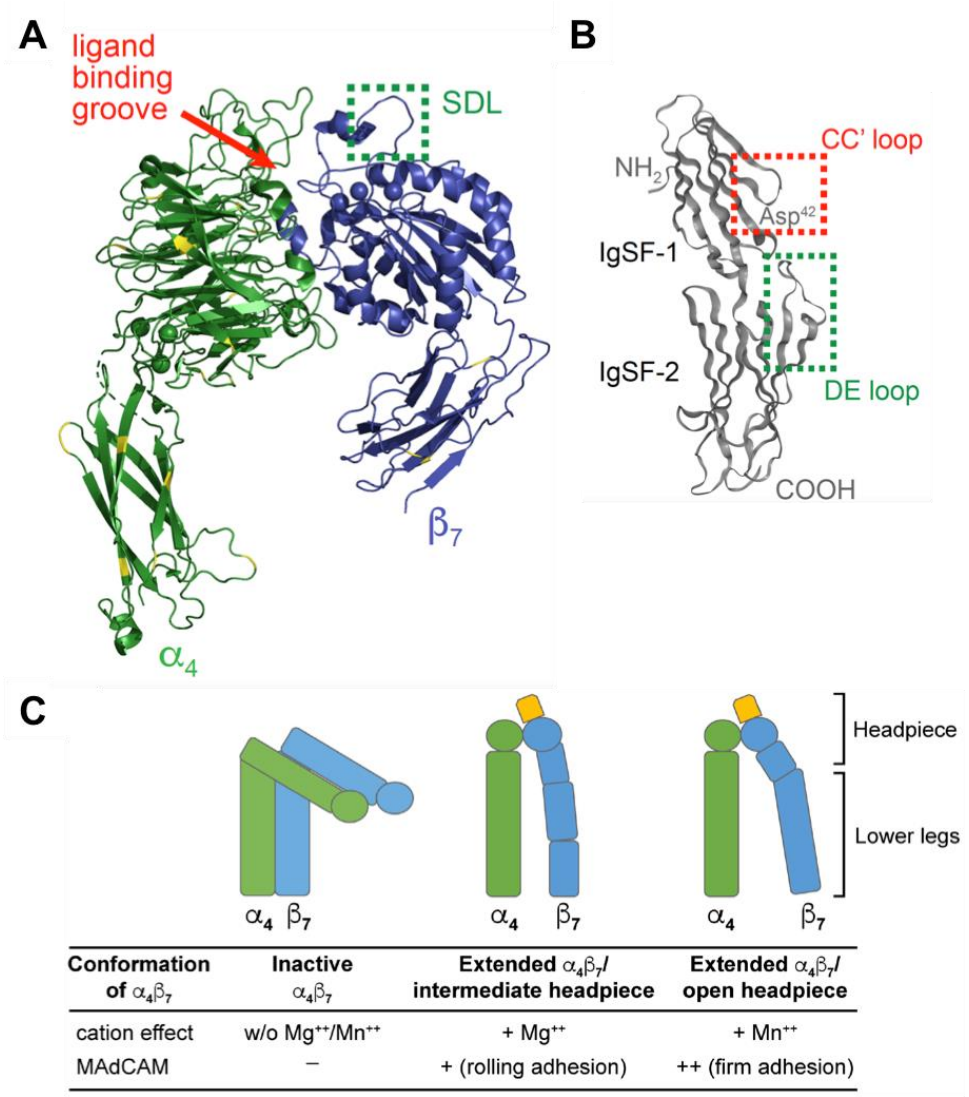
making a functional role for “non-functional” Env. This data also supports the connection between the gut-tropic nature of acute HIV infection and the interaction between $\alpha_4\beta_7$ and HIV Env.

3.2 Introduction

The gut associated lymphoid tissue (GALT) is the primary target of HIV infection and replication, particularly during the first weeks of infection [1, 2]. In the first few days of infection, high levels of replication take place in the GALT leading to severe depletion of $CD4^+$ T-cells in this compartment [2-4]. The destruction of GALT $CD4^+$ T-cells is associated with irreversible damage to the mucosal GALT barrier that is thought to play a major role in the chronic immune activation seen in HIV infection [5-7]. Even with administration of anti-retroviral therapy (ART) shortly after infection, this damage is not reversible [8]. While the early events of HIV infection significantly contribute to GALT destruction, much is still unknown about the early seeding of the GALT during acute HIV infection.

Our group and others have shown that recombinant HIV envelope protein (Env) binds to and signals through integrin $\alpha_4\beta_7$, a lymphocyte gut homing receptor [9-12]. Integrin $\alpha_4\beta_7$ is expressed on the surface of several lymphocyte subsets including naïve and memory $CD4^+$ T-cells [13]. Integrin $\alpha_4\beta_7$ is structurally dynamic in that it can adopt at least three distinct conformational states, possibly four. Of these conformational states, two are extended, and mediate lymphocyte rolling adhesion [14]. The transition between the conformations of $\alpha_4\beta_7$ are regulated intracellularly [15]. The normal function of $\alpha_4\beta_7$ in the multi-stage rolling adhesion cascade, is the binding to its natural ligands, mucosal addressin cellular adhesion molecule 1 (MAdCAM), vascular addressin cellular adhesion molecule 1 (VCAM) and the alternatively-spliced III connecting segment (CS) fragment of Fibronectin [16]. Integrin $\alpha_4\beta_7$ is the only integrin capable of binding to MAdCAM [17]. MAdCAM is expressed on follicular dendritic cells in the GALT [18, 19] as well as on the

endothelial cells that line the lumen of the high endotheial venules (HEVs) of the GALT and lamina propria of adults [20-22]. The tissue specific expression of MAdCAM and the specificity of MAdCAM for $\alpha_4\beta_7$ together define $\alpha_4\beta_7$ as the gut homing receptor.



Conformation of $\alpha_4\beta_7$	Inactive $\alpha_4\beta_7$	Extended $\alpha_4\beta_7$/ intermediate headpiece	Extended $\alpha_4\beta_7$/ open headpiece
cation effect	w/o Mg^{++}/Mn^{++}	+ Mg^{++}	+ Mn^{++}
MAdCAM	–	+ (rolling adhesion)	++ (firm adhesion)

Figure 3.1. Structure of $\alpha_4\beta_7$ heterodimer and MAdCAM binding motif. (A) Ribbon diagram of a human $\alpha_4\beta_7$ heterodimer headpiece (α_4: green, β_7: blue) (PDB ID: 3V4P). Ligand binding groove is highlighted by a red arrow, and the SDL is highlighted by a green box. (B) Ribbon diagram of the two N-terminal IgSF domains of human MAdCAM (PDB ID: 1GSM). The MAdCAM CC' loop of IgSF domain 1 is highlighted in the red box and the DE loop of IgSF domain 2 is highlighted in a green box. (C) Schematic of three conformations of the cell surface expression of the $\alpha_4\beta_7$ heterodimer. The influence of divalent cations (Mg^{++} and Mn^{++}) on the type of adhesion is listed below.

While $\alpha_4\beta_7$ is not required for viral entry for HIV [23-25], our findings suggest a link between the gut-tropic nature of HIV infection and this interaction. One suggested role is as an attachment factor [26]. While the direct role that $\alpha_4\beta_7$ plays in HIV infection is still unknown, growing evidence suggests that $\alpha_4\beta_7$ plays a significant role in the pathogenesis of HIV. It has been shown that $\alpha_4\beta_7^{high}CD4^+$ memory T-cells are preferentially infected and depleted early on in both HIV and SIV infection [8, 27]. Additionally, the pre-infection frequency of $\alpha_4\beta_7^{high}CD4^+$ memory T-cells is correlated with risk of acquisition of SIV in a NHP transmission model [28] and is correlated with risk of acquisition and disease progression of HIV in prospective cohort of women in Africa [8]. In an NHP low-dose intravaginal SIV transmission model, pre-infusion with an anti-$\alpha_4\beta_7$ mAb protected a significant proportion of animals from SIV infection and protected the GALT of the animals that did become infected [29]. Additionally, anti-$\alpha_4\beta_7$ mAb infusions combined with daily ART promoted durable control of viremia in SIV infected animals after all drug therapy was terminated [30]. These studies highlight the significant role $\alpha_4\beta_7$ likely plays in the gut-tropic nature of SIV and HIV transmission and pathogenesis, and underscores the need for greater understanding of the interaction between gp120 and $\alpha_4\beta_7$.

Our group and others have shown that a tripeptide motif at positions 179-181 (L^{179}, D^{180} and V/I^{181}) of the V2 domain of gp120 plays a major role in the interaction with $\alpha_4\beta_7$ [9, 10, 12]. The highly conserved LDV/I motif in gp120 is almost identical to the LDT motif in MAdCAM, the IDV motif in VCAM and the LDV motif in Fibronectin that are critical for binding $\alpha_4\beta_7$. The core aspartic acid in each of these $\alpha_4\beta_7$ ligands coordinates with a Mg^{++} ion that sits in the metal ion dependent adhesion site (MIDAS) of β_7, which is required for binding [14, 31]. Another, cryptic tripeptide motif involved in the interaction

was identified slightly upstream of the LDV/I motif at positions 170-172 (Q^{170}, R^{171}, and V^{172}) [12]. These two $\alpha_4\beta_7$ binding motifs are flanked by PNGSs, and removal of these sites enhances recombinant gp120 binding to $\alpha_4\beta_7$ [25]. This suggests that glycan carbohydrates may impact the interaction although it is not currently known how reduced glycans enhance binding. It is possible that glycans could impact the interaction by direct steric hinderance or through conformational constraints indirectly imposed by glycans.

In the context of recombinant, stabilized trimeric Env spikes, cryo-electron microscopy and X-ray diffraction analysis show that the V2 region, including positions 170-181, appears at the apex of the trimer [32-34] and is presented in a β–strand or β–barrel conformation [33-38]. In these structures, the LDV/I motif appears to be buried in a way that occludes binding of $\alpha_4\beta_7$. Because of this structural occlusion, we hypothesize that binding of $\alpha_4\beta_7$ to the V2 must involve an alternative presentation or a rearrangement of the binding motifs of V2. However, it is possible that due to the artificial nature of recombinant stabilized Env trimers and the fluidity in Env structure, that the $\alpha_4\beta_7$ binding site is not entirely occluded from the native trimer spike.

It has been shown that the V2 of gp120 is structurally polymorphic in that it can adopt a number of distinct conformations [39, 40]. In the context of the recombinant stabilized trimer, the V2 adopts a conformation where positions 170-181 are partially or entirely presented as a β–strand, notably the β–barrel introduced above or a Greek key conformation. These structural analyses utilize bnAbs in complex with SOSIP stabilized recombinant trimers or scaffold proteins grafted with the V1V2 loop. bnAbs that recognize these conformations tend to be broadly neutralizing, glycan dependent, and trimer preferring [41, 42]. Because the Env trimer is necessary for viral entry, it is believed that

the β–stranded conformation of the V2 is associated with this function. Other structural analyses using linear V2 peptides have shown that the V2 can adopt an alternative conformation that is helical or coiled. These analyses utilize what are known as V2p (peptide) mAbs, which are in general weakly or non- neutralizing and are glycan independent, to form complexes with linear V2 peptides [39, 40]. The mechanism by which the conformation of the V2 loop is structured, and the role of these alternative conformations is not known.

In this chapter, we characterize the physical interaction involved in the binding and adhesion of Env to $\alpha_4\beta_7$ and the conformational intricacies of the V2 for this interaction. We reason that such data provide insight into the role of $\alpha_4\beta_7$ in HIV pathogenesis. We demonstrate that the V2 loop of gp120 appears to mimic the way in which MAdCAM interacts with $\alpha_4\beta_7$. This mimicry may provide HIV a mechanism of gut-tropism which provides access to ideally activated target cells and may further the connection between the interaction with the gut homing receptor and the GALT damage associated with HIV pathogenesis. This brings forward the possibility that drugs that antagonize the interaction between MAdCAM and $\alpha_4\beta_7$ (i.e. FDA approved anti-$\alpha_4\beta_7$ mAb, Vedolizumab) could be used to antagonize the interaction between HIV Env and $\alpha_4\beta_7$. We show that select anti-V2 mAbs isolated from vaccine recipients and naturally infected individuals block the interaction between $\alpha_4\beta_7$ and HIV Env in a meaningful way. Several of these vaccine-elicited anti-V2 mAbs have been associated with protection from infection yet are weakly neutralizing. These V2p mAbs that block the interaction recognize an alternative form of Env not found in the recombinant stabilized Env trimer. Because glycosylation can greatly impact the structure and antigenicity of Env gp120, we also sought to characterize the Env

epitope that is recognized by these V2p mAbs. We report that this alternative helical/coiled conformation of the V2 is present on infected cells and virions and that it may originate from incompletely processed Env with reduced glycan processing. This suggests that immature or Env processed in a Golgi-independent pathway is incorporated into virions and may have an as yet unidentified functional role: facilitating the interaction between $\alpha_4\beta_7$ and HIV Env.

3.3 Experimental Procedures and Methods

3.3.1 Cell lines and reagents

RPMI8866 cells, a human B lymphoma cell line that constitutively expresses α4β7 was purchased from Sigma-Aldrich. Cells were maintained in RPMI-1640 (Lonza) containing 10% heat inactivated fetal bovine serum (Gibco), 2% penicillin/streptomycin/ glutamine (Gemini Bio-Products) and 0.1% retinoic acid (RA). Cells were cultured for a minimum of 7 days prior to use in adhesion assays. RA was obtained from Sigma Chemical. mAbs CH58, human mAb isolated from an RV144 vaccinated individual, and VRC01 were provided by the NIAID AIDS reagent program. CAP228-16H mAb was generated in the laboratory of Dr. Lynn Morris (CAPRISA) [43, 44]). The 830A mAb was provided by Dr. Susan Zolla-Pazner (Mt. Sinai Medical School) [35]. Cyclic V2 peptides with > 90% purity and having an amino-terminal biotin, derived from 92TH023, BG505 and C06980v0c22 were supplied by JPT Peptide Technologies. Scaffolds V1/V2 92TH023-1FD6 and V1/V2 92TH023-Tag were constructed, expressed and purified as described elsewhere [35]. CHO cell derived A244 gp120 (Lot 26539–1) was provided by Global Solutions for Infectious Diseases (San Francisco, CA). Purification employed an

anti-gD immunoaffinity resin, followed by both cation and anion exchange chromatography steps. Purity was estimated at 97.1%. BG505 SOISP trimer was generously provided by Dr. Paolo Lusso, LIR/NIAID. Vedolizumab was obtained from the NIH Clinical Center Pharmacy Department. Human integrin α4 mAb 2B4, MAdCAM-Ig, soluble $\alpha_4\beta_7$ and $\alpha_4\beta_1$ were obtained from R&D Systems. The LDV mimetic ELN-475772 was provided by ELAN Pharmaceuticals [45].

3.3.2 Deglycosylation of purified gp120 and gp140 peptides

Prior to use purified gp120 and gp140 proteins utilized in the $\alpha_4\beta_7$ adhesion assays were first treated with a deglycosylation protocol [46]. Purified proteins were treated with 500 U of PNGase F (NEB) per 20 µg of protein under non-denaturing conditions (1X GlycoBuffer 2 (NEB), 5mM DTT, and water) at 37 ºC for 3 h.

3.3.3 Surface plasmon resonance analysis of $\alpha_4\beta_7$ or anti-V2 loop mAb binding to Hu-MAdCAM, gp120 or cyclic V2 peptides

Experiments were performed using a Biacore 3000 instrument (GE Life Sciences) using CM4 or CM5 sensor chips. The data were evaluated using BIAevaluation 4.1 software (GE Life Sciences). The chip surface was activated by injecting 35 µl of a 1:1 mixture of 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N*-(dimethylaminopropyl) carbodiimide at 5 µl/min. NeutrAvidin, HIV gp120 or Hu-MAdCAM-Ig (R&D Systems) at concentrations of 5 µg/ml in 10mM NaOAc, pH 4.5, were immobilized to approximately 750 resonance units (RU). After the proteins were immobilized to the desired densities, unreacted sites on each surface were blocked with 35 µl of 1 M Tris-HCl (pH 8.0).

Biotinylated cyclic V2 peptides (1 μg/ml in 20 mM Tris-HCl, pH 8.0) were bound to the NeutrAvidin surfaces to densities of approximately 250–300 RU. One surface was activated and blocked without ligand to act as a control surface for non-specific binding of the soluble ligand. Any binding was subsequently subtracted from the remaining surfaces. Running buffer was HBS (pH 7.4), 0.01 mM $CaCl_2$, either 1 mM $MgCl_2$ or $MnCl_2$, 0.005% Tween-20, 0.05% soluble carboxymethyl-dextran. Binding experiments were carried out at a flow rate of 25 μl/min at 25 ºC. After a 2 min injection, the surface was washed for an additional 2 min in running buffer to follow dissociation of the bound ligand from the surface. The surfaces were regenerated by multiple injections of 4.5 M $MgCl_2$ at a flow rate of 100 μl/min. Inhibition of $\alpha_4\beta_7$ or anti-V2 loop antibodies by linear V2-loop peptides was carried out by pre-incubating the proteins with the peptides in running buffer at the indicated concentrations for 2 h at room temperature prior to passing them over the prepared surfaces as described above.

3.3.4 Kinetic analysis of anti-V2 loop mAbs binding to cyclic V2 peptides

Antibodies were diluted to the indicated concentrations in running buffer prior to being sequentially passed over the surface bound cyclic peptides as described above. The resulting sensorgram series were analyzed using the BiaEvaluation 4.1 software (GE Life Sciences) and fitted using a 1:1 Langmuir binding model to determine the kinetic rate and affinity constants.

3.3.5 $\alpha_4\beta_7$ adhesion to MAdCAM, gp120, and cyclic V2 peptides

The binding of $\alpha_4\beta_7$ expressed by the RPMI8866 cell line to MAdCAM, HIV A244 gp120, and HIV cyclic V2 peptides in the absence or presence of vedolizumab or 2B4 or ELN-475772 were analyzed by an adhesion assay (adapted from KK Peachman et al.) [11]. This assay was modified by culturing RPMI8866 cells in media containing 1 μM RA for at least 7 days prior to use in adhesion assays. Inclusion of RA increases adhesion to cV2 peptides, gp120 and MAdCAM [47]. Briefly, triplicate wells of a 96-well flat-bottom polypropylene plate (Greiner Bio-One) were coated overnight at 4 ºC with 100 μl of 2 μg/ml of MAdCAM (R & D Systems) or 100 μl of 2 μg/ml NeutrAvidin or 100 μl of 0.5–2.0 μg of deglycosylated HIV gp120 diluted in 50mM bicarbonate buffer, pH 9.6. The NeutrAvidin-coated plates were then incubated with biotinylated cyclic V2 peptides (5 μg/ml in bicarbonate buffer) for 1 h at 37 ºC. The solution from the plates was discarded and the plates were then blocked with blocking buffer (25 mM Tris, 2.7 mM KCl, 150 mM NaCl, 0.5% BSA, 4 mM $MnCl_2$, pH 7.2) for 1 h at 37 ºC. The solution was discarded, and plates were manually washed 4 times with blocking buffer. After blocking and washing the plate, RPMI8866 cells in a volume of 50 μl/well were pre-incubated for 40 min at 37 ºC with sample buffer in the absence or presence of 10 μg/ml of vedolizumab ($\alpha_4\beta_7$ mAb) or 2B4 (α_4 mAb) or ELN-475772 ($\alpha_4\beta_7/\alpha_4\beta_1$ dual inhibitor). Plates were then incubated with 50 μl/well of $2x10^5$ RPMI8866 cells at 37 ºC (5% CO_2) for 1 h, washed 5 times with PBS followed by the addition of 100 μl of RPMI-1640 containing 1% FBS, 1% penicillin/streptomycin/glutamine, 25mM HEPES with 10 μl/well of AlamarBlue dye. Fluorescence (excitation at 560 nm and emission at 590 nm) was kinetically measured immediately after the addition of the AlamarBlue dye for 8 h.

3.3.6 Capture of 92TH023 virions by MNPs coated with gp120 mAbs or $\alpha_4\beta_7$

92TH023 virions were captured with 15 nm magnetic nanoparticles (MNPs) coupled to $\alpha_4\beta_7$ or 2G12, CH58, PG9, and 830A mAbs as previously described [48]. Briefly, carboxyl-terminated iron oxide nanoparticles (Ocean Nanotech, San Diego) via two step carbodiimide reaction were coated with 1 mg of anti-gp120 mAbs or recombinant soluble $\alpha_4\beta_7$ according to manufacturer's protocol. Virus preparations were derived from RA activated primary PBMCs infected with an infectious molecular clone derived from 92TH023, using standard conditions. To capture virions, MNPs coated with mAbs ($3.9x10^{12}$) in 60 µl were incubated with viral preparation (33 ng/ml based on viral p24 content) for 1 h at 37 ºC. Captured virions were separated on MACS magnetic columns attached to an OctoMacs magnet (Miltenyi Biotech), washed 4 times with 600 µl wash buffer (0.5% bovine serum albumin, 2 mM EDTA in PBS), eluted in 200 µl PBS, and analyzed on Luminex X200 for p24 content using a dynamic immunofluorescent cytometric bead assay [49]. In experiments with $\alpha_4\beta_7$-MNPs, virions were incubated and washed with complete medium with 1 mM $MnCl_2$.

3.3.7 Inhibition of $\alpha_4\beta_7$ adhesion to gp120 and cyclic V2 peptides by V2 mAbs

Triplicate wells of a 96-well flat-bottom polypropylene plate (Greiner Bio-One) were coated with biotinylated cyclic V2 peptides or deglycosylated gp120 as described above. After blocking and washing, plates were incubated with 20–100 µg/ml of the designated anti-V2 mAbs in sample buffer (25 mM Tris, 2.7 mM KCl, 150 mM NaCl, 4 mM $MnCl_2$, 1% FBS, pH 7.2) for 1 h at 37 ºC. RPMI8866 cells were pre-incubated for 40

min at 37 ºC with sample buffer. Plates were then incubated with 50 µl/well of $2x10^5$ cells at 37 ºC (5% CO_2) for 1 h, washed 5 times with PBS, followed by the addition of 100 µl of RPMI-1640 containing 1% FBS, 1% penicillin/streptomycin/glutamine, 25 mM HEPES and 10 µl/well of AlamarBlue dye (Invitrogen). Following the addition of AlamarBlue dye (excitation at 560 nm and emission at 590 nm) fluorescence was measured kinetically for a period of 8 h.

3.3.8 $\alpha_4\beta_7$ adhesion to constrained and unconstrained V1V2 92TH023 scaffolds

92TH023 V1/V2 was cloned into both 1FD6 (constrained) and tag (unconstrained) scaffolds. Plates were then coated with 0.5, 1.0, and 2.0 µg of either V1/V2 92TH023 1FD6 or V1/V2 92TH023 tag scaffolds followed by addition of RPMI8866 cells. The plates were washed and 100 µl of media and 10 µl of AlamarBlue dye was added to each well as described above. Fluorescence (excitation at 560 nm and emission at 590 nm) was measured kinetically after the addition of the AlamarBlue dye.

3.3.9 Surface staining of HIV infected $CD4^+$ T-cells by flow cytometry

$CD4^+$ T-cells were isolated from healthy donor PBMCs using a negative selection isolation kit as prescribed (Stem Cell Technologies). Cells were activated with OKT3, IL-2 and retinoic acid for 4 days before infection. Cultures were inoculated with infectious molecular clones generated using the designated isolate Env sequence (92TH023, CMU06, BG505, AA05, and SF162) inserted into an NL4.3 viral backbone. Viral constructs were first transfected into 293T cells before a single passage through PBMCs and harvested. Infected $CD4^+$ T-cells were washed with PBS with 5% FBS and stained with 1 µg of anti-

Env mAb (CH58, CH59, or PG9) for 3 h at 4 °C. Cells were washed and then stained with fluorescently labeled secondary goat anti-human Ab for 30 min at 4°C. Samples were then washed and fixed in 4% paraformaldehyde, acquired using a BD Canto II flow cytometer and analyzed using FlowJo software.

3.4 Results

3.4.1 The affinity of $\alpha_4\beta_7$ for gp120 V2 is similar to its affinity for MAdCAM

Previous studies have demonstrated the affinity of the specific interaction between gp120 and $\alpha_4\beta_7$ in a qualitative way [9-12]. We sought to determine if the interaction between gp120 and $\alpha_4\beta_7$ was in apparent mimicry of the interaction between MAdCAM and $\alpha_4\beta_7$ by measuring the specific affinities between the two sets of interacting proteins. To do so, we carried out an SPR based assay that utilized dextran surfaces coated with recombinant Env peptides and soluble $\alpha_4\beta_7$ heterodimer. Soluble $\alpha_4\beta_7$ was passed over surfaces coupled with either Env peptides or MAdCAM-Ig fusion protein. Using a recombinant gp120 from isolate A244 (subtype A/E) and MAdCAM-Ig in the presence of $MnCl_2$-containing buffer, we measured similar specific affinities for $\alpha_4\beta_7$ (K_D of 7.140 and 0.597 nM, respectively) (Figure 3.2). These affinities were found to be generally comparable to that of gp120 and soluble CD4 (K_D of 22 nM) [50]. We next replaced the A244 recombinant gp120 with a cyclic V2 42-amino acid peptide (cV2) derived from the 92TH023 isolate (subtype A/E). The affinity of this cV2 92TH023 for $\alpha_4\beta_7$ was found to be similar to that of the A244 recombinant gp120 (K_D of 1.150 nM) (Figure 3.2) suggesting that the V2 of gp120 is sufficient to mediate the high affinity interaction between Env and $\alpha_4\beta_7$. Since the cV2 peptide does not contain any N-linked glycans or co-purified

contaminants, this suggests that the interaction does not require glycans or matrix proteins [51]. We also extended this analysis to a cV2 peptide from the C06980v0c22 isolate (subtype C) and recombinant gp120 from BG505 (subtype A) isolate (K_D of 1.580 and 0.919 nM, respectively) (Figure 3.2). Together, this data suggests that the interaction between HIV Env and $\alpha_4\beta_7$ is highly conserved and has a comparable affinity to that of the natural ligand, MAdCAM.

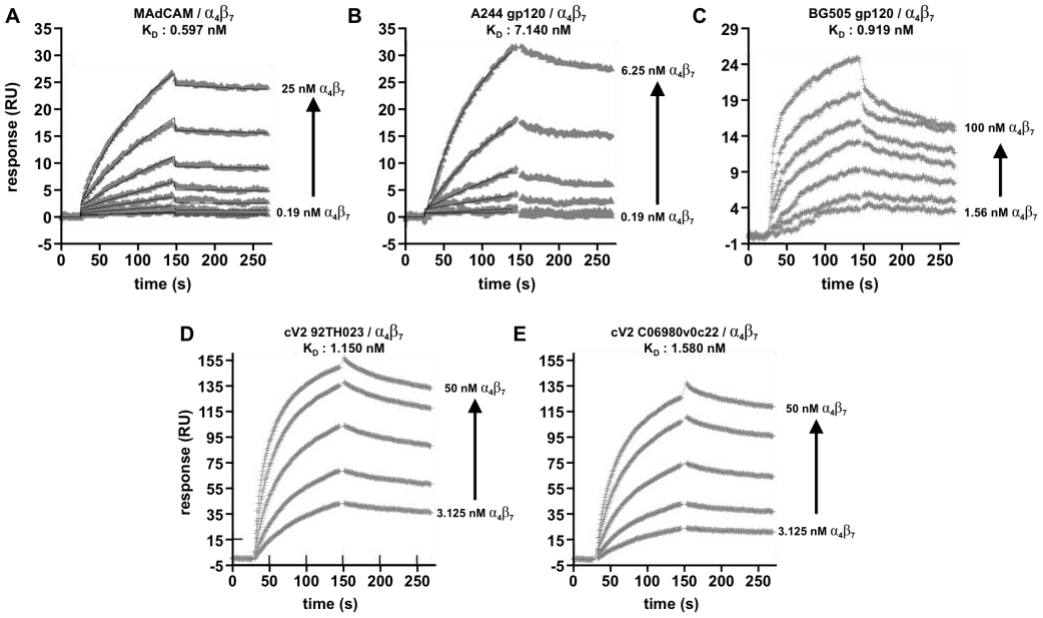


Figure 3.2. Specific affinity of $\alpha_4\beta_7$ for MAdCAM, HIV gp120s and cV2 peptides. (A) Sensorgram of increasing concentrations (2-fold) of soluble $\alpha_4\beta_7$ passed over surface-immobilized MAdCAM in the presence of 1 mM $MnCl_2$ for 120 sec, followed by a 120 sec washout/dissociation phase. Mass of bound $\alpha_4\beta_7$ (y-axis) expressed as response units (RU). Affinity expressed as K_D. (B) Same as in panel A, with immobilized recombinant A244 gp120. (C) Same as in panel A, with immobilized recombinant BG505 gp120. (D) Same as in panel A, with immobilized cyclic V2 (cV2) 92TH023 peptide. (E) Same as in panel A, with immobilized cV2 C06980v0c22 peptide. Each sensorgram is representative of three independent measurements.

3.4.2 Adhesion of $\alpha_4\beta_7$ to gp120 is inhibited by antagonists of the MAdCAM - $\alpha_4\beta_7$ interaction

The similar high affinities of gp120, cV2 peptides, and MAdCAM for $\alpha_4\beta_7$ suggest that gp120 is mimicking the way in which MAdCAM interacts with $\alpha_4\beta_7$. Several

antagonists of the interaction between MAdCAM and $\alpha_4\beta_7$ have been developed to treat inflammatory bowel disease (IBD) by blocking the MAdCAM binding site on $\alpha_4\beta_7$. If the interaction between gp120 and $\alpha_4\beta_7$ is mediated by the same mechanism, MAdCAM binding antagonists could be used to interrupt gp120 engagement of $\alpha_4\beta_7$.

To test the ability of these MAdCAM antagonists to inhibit $\alpha_4\beta_7$ adhesion to gp120, we performed a slightly modified adhesion assay based on the protocol developed by Peachman and colleagues [11]. In this static adhesion assay, retinoic acid activated $\alpha_4\beta_7$-expressing RPMI8866 cells were incubated with MAdCAM antagonists. Then the cells were added to a 96-well plate coated with MAdCAM or Env peptides. After incubation, the non-adhered cells were washed away, and adherence was measured by fluorescent cellular dye. As expected, the MAdCAM antagonists inhibited adhesion of $\alpha_4\beta_7$-expressing cells to MAdCAM coated wells (Figure 3.3). Adhesion was inhibited by an FDA approved mAb therapy used to treat IBD, vedolizumab, which inhibits MAdCAM binding by targeting the specificity determining loop (SDL) of β_7. Adhesion was also inhibited by the small molecule mimetic of the LDV motif, ELN-475772, which blocks the interaction by binding to key residues in the ligand binding groove of $\alpha_4\beta_7$. We then tested if these MAdCAM antagonists could inhibit the adhesion of $\alpha_4\beta_7$ to HIV Env using the cV2 92TH023 peptide described above in our adhesion assay. We found that the anti-α_4 mAb 2B4, soluble MAdCAM, ELN-475772, and vedolizumab could all inhibit adhesion of $\alpha_4\beta_7$-expressing cells to the cV2 92TH023 peptide (Figure 3.3).

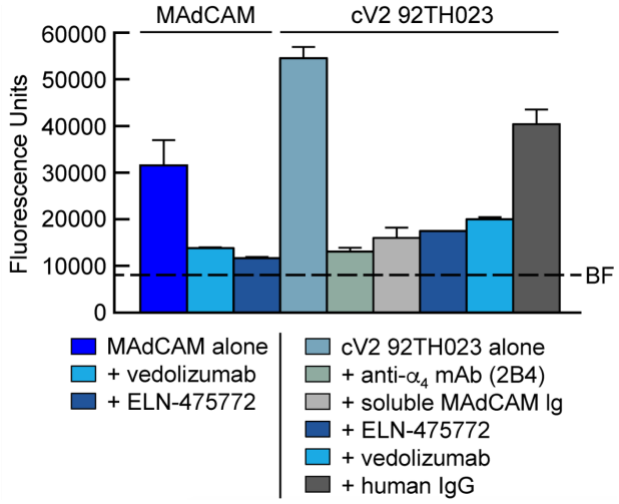


Figure 3.3. Antagonists of MAdCAM engagement with $\alpha_4\beta_7$ inhibit adhesion of $\alpha_4\beta_7$-expressing cells to cyclic V2 peptides. A representative result of RPMI8866 cells adhering to immobilized MAdCAM or a cV2 92TH023 peptide in the absence or presence of vedolizumab, an LDV mimetic ELN-475772, the anti-α_4 specific mAb 2B4, soluble MAdCAM-Ig, or control human IgG, as indicated. Adhesion was determined at OD 590 nm and listed as fluorescence units (y-axis). Background fluorescence (BF) of RPMI8866 cell adhesion to blank wells is denoted by a dashed line. Adhesion to immobilized MAdCAM serves as a specificity control for $\alpha_4\beta_7$ adhesion and human IgG is employed as a reagent control. Conditions are run in triplicate and error bars indicate standard error of the mean (SEM).

3.4.3 Adhesion of $\alpha_4\beta_7$ is mediated by a reduced glycan form of gp120 v2

We have previously reported that removal of N-linked glycosylation sites, particularly those of the V2, can influence the capacity of $\alpha_4\beta_7$ to mediate binding of gp120 in a flow cytometric assay [25]. Rao and collogues recently reported that the adhesion of recombinant gp120 to $\alpha_4\beta_7$ requires partial de-glycosylation by gentle enzymatic removal of N-linked glycans [46].

To determine the importance of N-linked glycosylation in our functional adhesion assay, we used the recombinant gp120 from A244 isolate to test its capacity to mediate adhesion of $\alpha_4\beta_7$-expressing cells to a multivalent surface. This gp120 does not require modification to mediate binding of $\alpha_4\beta_7$ in our SPR assay shown above. However, as suggested above by us and others, we found that gentle de-glycosylation of the A244 gp120

was necessary to mediate adhesion of $\alpha_4\beta_7$-expressing cells (Figure 3.4). This dependency for glycan trimming is in agreement with our previous work showing that removal of V2 glycans can increase binding of recombinant gp120 to $\alpha_4\beta_7$ [25].

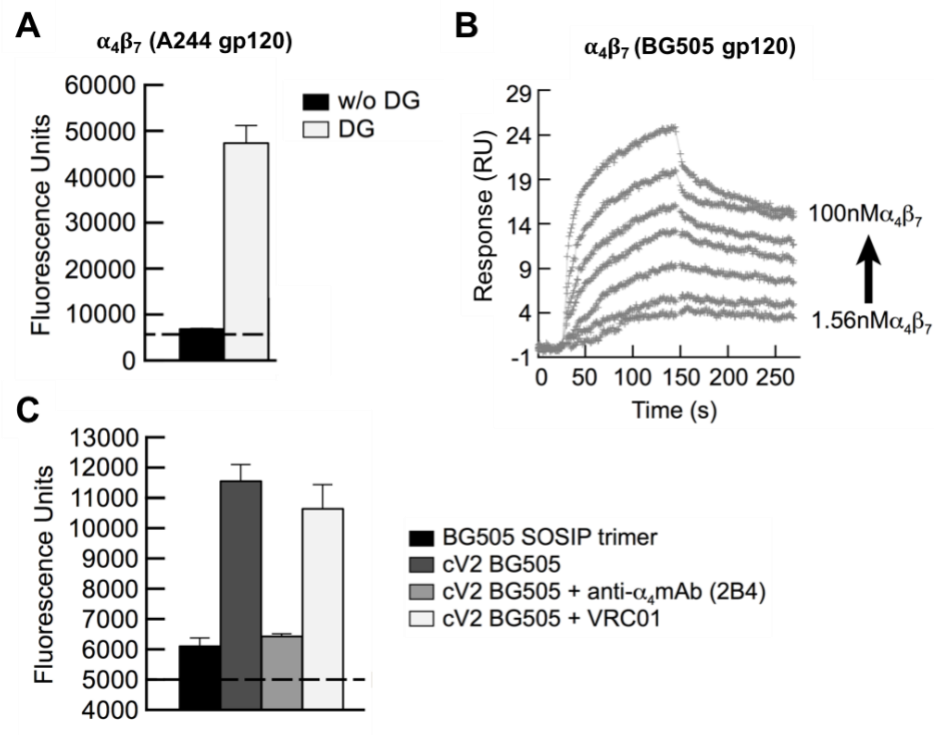


Figure 3.4. Adhesion of $\alpha_4\beta_7$ to deglycosylated gp120 and recombinant trimer. (**A**) Adhesion of RPMI8866 cells to immobilized A244 gp120. Deglycosylation (DG) of gp120 as indicated. (**B**) Sensorgram of increasing concentrations (2-fold) of soluble $\alpha_4\beta_7$ passed over surface-immobilized BG505 gp120 in the presence of 1 mM MnCl2 for 120 sec, followed by a 120 sec washout/dissociation phase. Mass of bound $\alpha_4\beta_7$ (y-axis) expressed as response units (RU). (**C**) Comparison of adhesion of RPMI8866 cells to BG505 SOSIP vs. cV2 BG505 peptide. The anti-α4 mAb 2B4 is employed as a positive control and the gp120 mAb VRC01 is employed as a negative control.

We also evaluated the ability of a SOSIP stabilized recombinant Env trimer from the BG505 isolate to mediate adhesion of $\alpha_4\beta_7$-expressing cells. We found that even though the recombinant BG505 gp120 can mediate binding to soluble $\alpha_4\beta_7$ in our SPR assay (Figure 3.4), the BG505 SOSIP trimer was unable to mediate adhesion of $\alpha_4\beta_7$-expressing cells even after gentle de-glycosylation (Figure 3.4). Given that the recombinant BG505

gp120 can bind $\alpha_4\beta_7$ but the BG505 trimer cannot mediate adhesion, we hypothesize that the conformational constraints on the V2 in the context of the trimer precludes the presentation of the $\alpha_4\beta_7$ binding epitope.

Taken together this data suggests that glycosylation can impact the interaction between the V2 and $\alpha_4\beta_7$ in a major way, but structural characteristics also play a role in the manner in which the $\alpha_4\beta_7$ binding sites on V2 are presented.

3.4.4 Weakly neutralizing anti-V2 mAbs elicited by vaccination and natural infection inhibit gp120-$\alpha_4\beta_7$ adhesion

The conformation of the V2 domain of gp120 is dynamic and structurally heterogeneous in that it can adopt a range of distinct conformations. Because of this, portions of the V1V2 loop of gp120 were often removed from recombinant proteins used to generate high resolution crystal structures [52, 53]. To obtain the structure of the V1V2 loop, the V1V2 fragment has been grafted onto scaffolds derived from unrelated proteins. These scaffolds stabilize the V1V2 fragment in complex with conformation dependent V2 mAbs capable of generating high resolution structures. One such scaffold, 1FD6, grafted with a V1V2 fragment in complex with the glycan-dependent bnAb PG9 showed that the V2 can adopt a β-sheet conformation in a Greek key structure [36]. The same 1FD6/V1V2 fragment was also complexed with the mAb 830A which showed that the V2 can also adopt a similar β-sheet conformation as part of a β-barrel structure [35]. This β-barrel conformation is consistent with reported crystal structures of pre-fusion Env trimer spikes [33, 34].

However, linear V2 peptides, which do not have the same constraints as scaffolded fragments, can adopt helical or coiled conformations [54]. This is the case for a linear V2 peptide in complex with the weakly neutralizing mAb CH58 [55]. This is noteworthy because mAb CH58 was isolated from an uninfected immunized individual enrolled in the mildly protective RV144 HIV Vaccine Trial. CH58 recognizes an epitope that maps to position 168-181 of the V2 loop. This region includes the two RV144 sieve analysis sites K^{169} and I^{181} which were identified as targets of vaccine elicited immune pressure in the RV144 trial. Helix-preferring mAbs have also been isolated from HIV-infected individuals. One such mAb, CAP228-16H, recognizes a helix-coil that is strikingly similar to that recognized by CH58 [43, 44].

Therefore, mAbs that recognize the same region of the V2 loop (positions 153-194) recognize at least two distinct epitopes: a constrained β-sheet recognized by glycan-dependent mAbs like PG9 and 830A, and a less constrained helix-coil conformation recognized by weakly neutralizing mAbs CH58 and CAP228-16H. While trimeric Env spikes, which are involved in viral entry, present a β-sheet conformation of V2 when stabilized in recombinant proteins, the function or significance of the helical conformation is not yet known.

To determine if these two groups of mAbs (830A β-sheet versus CH58 helix) can inhibit the adhesion of $\alpha_4\beta_7$-expressing cells to V2 peptides, we tested them in the adhesion assay described above. We found that the weakly neutralizing mAbs CH58, CH59 and CAP228-16H inhibited adhesion by >90% while bnAb 830A failed to inhibit adhesion to a detectable level (Figure 3.5). This suggests that the conformation in which the V2

mediates $\alpha_4\beta_7$ adhesion is not a constrained β-sheet conformation associated with the recombinant Env trimer, rather it is likely an alternative helical conformation.

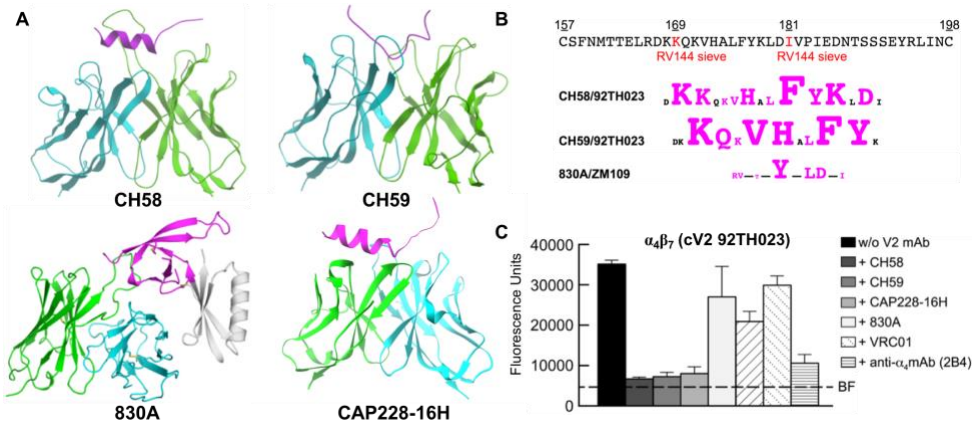


Figure 3.5. The structure of anti-V2 mAbs in complex with V2 peptides, and anti-V2 mAb inhibition of $\alpha_4\beta_7$ adhesion. (A) Crystal structure of four V2 domain mAbs, CH58 (PDB ID: 4HPO), CH59 (PDB ID: 4HPY), 830A (PDB ID: 4YWG), and CAP228-16H (PDB ID: 6FY2) in complex with V2 peptides, Only the Fv regions are shown with the heavy chain, light chain, and the V2 epitope colored cyan, green, and magenta, respectively. B) Amino acid sequence of the V2 domain of 92TH023. Sieve residues identified in the RV144 vaccine study are highlighted in red, along with schematics of the epitopes of CH58, Mk16C2 and 830A aligned below (with the corresponding HIV isolate listed). Residues in contact with each mAb are highlighted in magenta and the size of each amino acid is proportional to the contact surface area. C) Adhesion of RPMI8866 cells to cV2 92TH023 in the absence or presence of V2 mAbs: CH58, CH59, CAP228-16H, and 830A. The mAb VRC01 is included as a nonspecific mAb control, and the anti-α_4 mAb 2B4 is employed as a positive control. Adhesion was determined at OD 590 nm and listed as % inhibition in three or more independent experiments relative to cV2 92TH023 in the absence of any inhibitor (y-axis). Error bars indicate SD.

3.4.5 $\alpha_4\beta_7$ adhesion is mediated by an unconstrained form of the gp120 V2 loop

V2 helix-preferring mAbs inhibit adhesion of $\alpha_4\beta_7$-expressing cells, while V2 mAbs with overlapping residues but a trimer associated β-sheet epitope do not mediate this adhesion (Figure 3.5). Given this, and the failure of BG505 SOSIP trimer to meditate adhesion (Figure 3.4), we hypothesized that the Env trimer has underlying conformational restrictions that preclude the presentation or accessibility of the $\alpha_4\beta_7$ binding epitope.

To determine if the β-sheet conformation of the V2 is compatible with $\alpha_4\beta_7$ engagement, we grafted the V1V2 fragment of isolate 92TH023 into two separate scaffolds. The first scaffold, termed 1FD6, has been shown to present the V1V2 loop in a way that biases its conformation towards that of the β-barrel [35]. This V1V2-1FD6 scaffold is reactive with mAb 830A and bnAb PG9. The second scaffold, termed tags, has been shown to present the V1V2 untethered at the C-terminus allowing it to adopt an unconstrained, CH58-reactive helix conformation. We found that the 92TH023 V1V2-tags showed ~5x greater adhesion than did 92TH023 V1V2-1FD6 which only mediated a low level of $\alpha_4\beta_7$ adhesion (Figure 3.6). This data suggests that $\alpha_4\beta_7$ adhesion requires flexibility in the V1V2 loop that is precluded in the SOSIP stabilized BG505 trimer. This preclusion is likely due to structural constraints by neighboring sequences in the closed trimer.

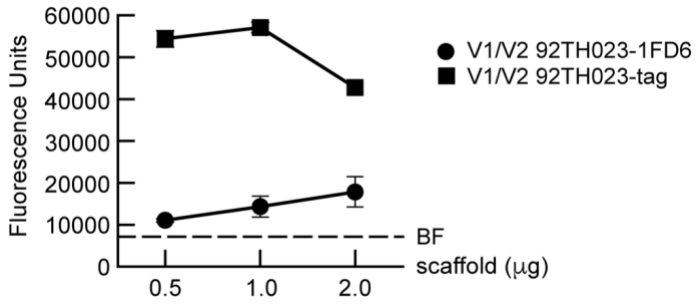


Figure 3.6. Adhesion of $\alpha_4\beta_7$ to V1V2 scaffolds. Comparison of adhesion of RPMI8866 cells to V1/V2 92TH023-1FD6 vs. V1/V2 92TH023-tag scaffolds using increasing concentrations of each scaffold as indicated. Adhesion was determined by OD 590 nm and listed as fluorescence units (y-axis). Conditions were run in triplicate and error bars indicate SEM. Results are representative of three independent experiments.

3.4.6 The unconstrained, helical conformation that mediates $\alpha_4\beta_7$ adhesion is found on incompletely processed gp120, and is present on the surface of infected cells and virions

HIV is unusual in that a significant fraction of expressed Env proteins appear to bypass the Golgi on their way to the cell surface [56]. This form of Env, termed ER-Env, lacks Golgi-specific modifications. Despite the lack of Golgi processing, ER-Env is still transported to the surface of the infected cell and is presumably incorporated into virions. While completely processed Env is believed to be required for viral entry, no function has been identified for ER-Env. One suggested possible function of ER-Env is immune evasion. Data described here and previously show that Env with reduced glycosylation mediates increased $\alpha_4\beta_7$ adhesion. ER-Env lacks large, complex glycan modifications. Therefore, we hypothesized that ER-Env may have a biased presentation of the V2 loop of gp120 that could engage $\alpha_4\beta_7$. To test this, we first probed the surface of $CD4^+$ T-cells infected separately with two infectious molecular clones (IMCs) derived from the 92TH023 and the CMU06 isolate with anti-V2 mAbs. We found that mAbs CH58 and CH59 reacted with Env on the surface of 92TH023 and CMU06 (subtype A/E) infected $CD4^+$ T-cells (Figure 3.7). This suggests that the unconstrained, helical conformation is present on the surface of infected cells. We then sought to determine whether the unconstrained, helical conformation was readily presented on ER-Env. To test this, we trypsinized infected $CD4^+$ T-cells to remove surface proteins, and then cultured them overnight in the absence or presence of Brefeldin A (BreA), an inhibitor of intracellular trafficking between the Golgi and ER compartments. Infected cells cultured in media containing BreA will likely have an increased proportion of ER-Env because only surface

proteins that are processed in a Golgi-independent pathway will make it to the cell surface. When we probed cells infected with viruses derived from four isolates (BG505, CMU06, SF162 and AA05), we found that the unconstrained, helix epitope recognized by CH58 was largely resistant to BreA (Figure 3.7). The β-sheet conformation of the Greek key structure recognized by the bnAb PG9 was, as expected, absent in the BreA condition. This suggests that the helical CH58 epitope is presented on incompletely processed, ER-Env.

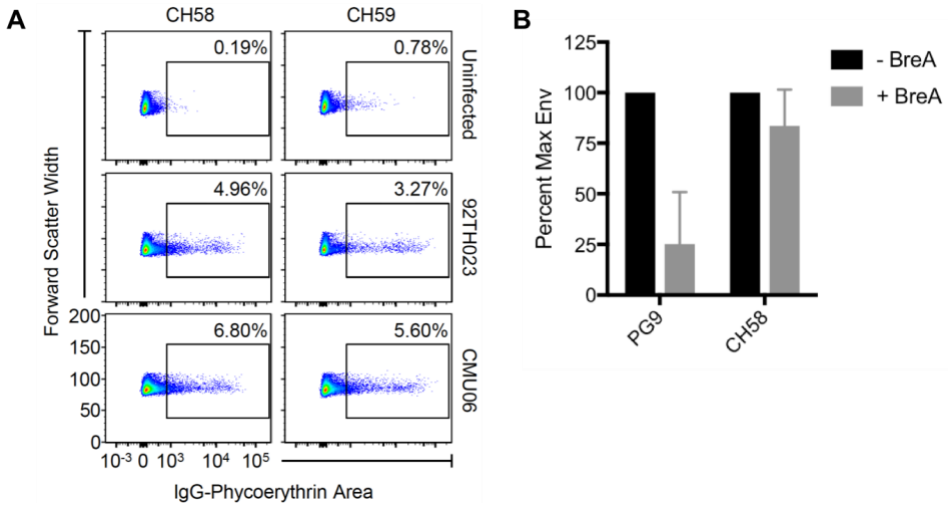


Figure 3.7. Helical, coiled conformation of the V2 loop of gp120 on the surface of infected cells. (A) Surface staining with mAbs CH58 and CH59 of primary $CD4^+$ T-cells infected with two isolates of HIV (92TH023 and CMU06; subtype A/E) or not infected. **(B)** Percent of mAb reactive Env on the surface of primary $CD4^+$ T-cells separately infected with four HIV isolates. Infected cells were trypsinized followed by overnight culture in the absence or presence of Brefeldin A (BreA). The Percent Max Env is calculated by normalizing the percent positive for each mAb to its matching BreA minus condition. Data shown is compilation of 2 or more experiments for each of the four viral isolates (AA05, CMU06, Bal and BG505).

We then sought to determine whether $\alpha_4\beta_7$-reactive Env and/or the helical conformation recognized by V2p mAbs that inhibit $\alpha_4\beta_7$ adhesion (i.e. CH58, CH59 and CAP228-16H), could be found on the surface of virions. To test this, we employed magnetic nanoparticles (MNPs) coated with either $\alpha_4\beta_7$ or V2 mAbs CH58, PG9, and 830A to capture 92TH023 virions derived from primary PBMCs isolated from a healthy donor.

The mAb 2G12 was employed as a positive control. Viral supernatants were incubated with V2 mAb-coated MNPs. After extensive washing, virion capture was measured by a Luminex-based p24 detection assay [49]. In three independent experiments CH58, PG9, and 2G12 each captured ~5X greater amounts of virus than non-specific IgG. Capture by mAb 830A was surprisingly less efficient (~3X over IgG) (Figure 3.8). The ability of mAb CH58, which recognizes a helical structure and inhibits V2 adhesion to $\alpha_4\beta_7$, to capture virions suggests that these virions may present an $\alpha_4\beta_7$-reactive form of Env. To test this directly we incubated virions with $\alpha_4\beta_7$-MNPs in the absence or presence of increasing amounts of the $\alpha_4\beta_7$ inhibitor ELN-475772. The $\alpha_4\beta_7$-MNPs captured virus to levels comparable to capture by mAbs 2G12, PG9 and CH58. This capture was inhibited by ELN-475772 in a dose-dependent manner (Figure 3.8). This data shows that 92TH023 virions derived from primary PBMCs present the V2 in a way that is reactive with both mAb CH58 (helical conformation) and $\alpha_4\beta_7$. Remarkably, this capture is comparable to the level of capture by mAb 2G12, which has been shown in protein G- and MNP-based virion capture assays to capture ~99% of virions in viral supernatants [57, 58]. This suggests that a significant fraction of virions express at least one gp120 that is reactive with CH58 and $\alpha_4\beta_7$.

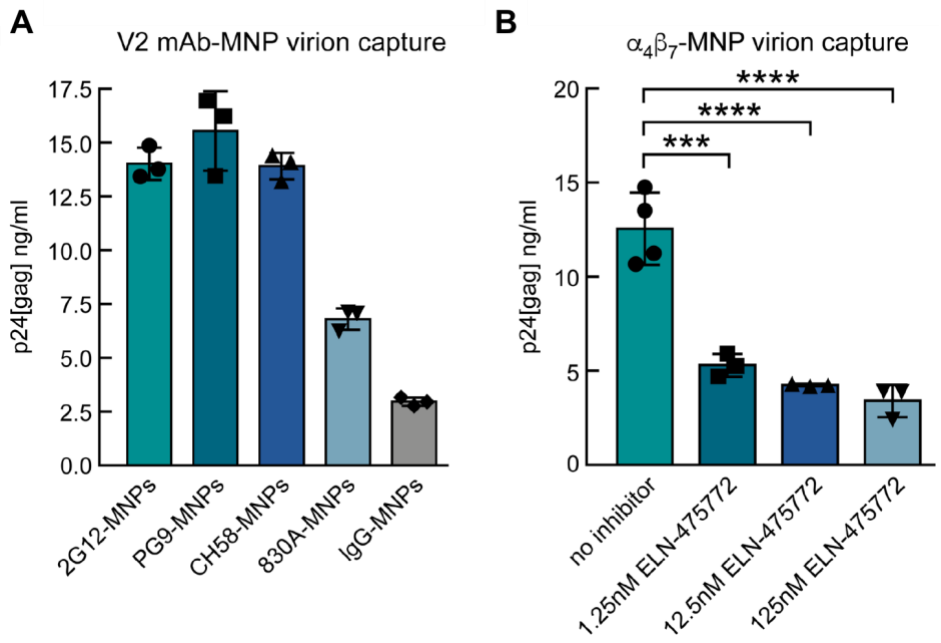


Figure 3.8. Virion capture by V2 mAbs and $\alpha_4\beta_7$ coated nanoparticles. A) Capture of 92TH023 virions by MNPs coated with gp120 mAbs including 2G12 (positive control) and V2 mAbs PG9, CH58 and 830A. A non-specific Ig was employed as a negative control. Virion capture was determined by detection of MNP-bound p24 (Y-axis). Results from three independent experiments are shown. B) 92TH023 virion capture by $\alpha_4\beta_7$-MNPs in the absence or presence of increasing concentrations of ELN-475772. Results from three independent experiments are shown. Detection of MNP-captured virus as in panel A. Error bars indicate SEM. Significance measure by ANOVA followed by the Tukey multiple comparison test (***p <0.001 and ****p <0.0001).

3.4.7 A 15-amino acid linear V2 peptide inhibits binding of $\alpha_4\beta_7$ to gp120

Liao and colleagues showed that a 15 aa peptide corresponding to residues 168-181 of the V2 loop adopts a helical structure when complexed with the weakly neutralizing mAb CH58 [55]. We investigated whether a similar 15 aa peptide corresponding to the V2 loop could inhibit the binding of $\alpha_4\beta_7$ to the V2 loop using the SPR binding assay described above. Soluble $\alpha_4\beta_7$ was passed over a cV2 92TH023 coated surface in the absence or presence of 8 overlapping linear 15 aa peptides that span the V2 loop. These peptides correspond to sequences in an HIV-1 subtype B consensus V2 domain (Figure 3.9A). Peptide H43 (Q^{170}KEYALFYKLDVVPI^{184}), which closely aligns with the peptide

employed by Liao and colleagues, inhibited $\alpha_4\beta_7$-binding by > 90% (Figure 3.9B). This peptide includes both the canonical L^{179} D^{180} V^{181} $\alpha_4\beta_7$ binding site and a Q^{170} K^{171} E^{172} motif that aligns with the $Q^{170}R^{171}V^{172}$ cryptic $\alpha_4\beta_7$ epitope identified by Cardozo and colleagues [12]. The peptides that flank H43, peptide H42 (R^{166} DKVQKEYALFYKLD^{180}) containing just the QKE but not the full LDV motif, and peptide H44 (A^{174}LFYKLDVVPIDNDN^{188}), containing just the LDV but not the QKE motif, do not inhibit binding. This suggests that the 15 aa V2 peptide containing the two $\alpha_4\beta_7$ epitopes is sufficient to inhibit the interaction while 15 aa peptides that contain only one of the epitopes are not.

We repeated this analysis but substituted cV2 92TH023 with A244 gp120 and obtained a similar result (Figure 3.9C). We then competed mAbs CH58 and CAP228-16H with these same peptides (Figure 3.9D and E). Peptides H42 and H43 each partially inhibited mAb CH58 binding and strongly inhibited (> 90%) CAP228-16H binding. This data supports the finding that the CH58 and CAP228-16H epitopes overlap with the $\alpha_4\beta_7$ epitope.

Taken together these results suggest that inhibition by peptide H43 involves direct binding to $\alpha_4\beta_7$. To rule out allosteric inhibition (i.e. peptide H43 binding directly to, and altering the conformation of V2), we carried out a similar peptide inhibition assay with immobilized MAdCAM and determined that H43 inhibited $\alpha_4\beta_7$ binding to MAdCAM by > 90% (Figure 3.9F). This result is best explained by direct competition between the L^{179}-D^{180} in peptide H43 and the critical L^{41}-D^{42} encoded within MAdCAM IgSF domain 1 (Figure 3.9G). However, we believe it is very likely that other residues in H43 also engage $\alpha_4\beta_7$. We conclude that an epitope contained within a linear peptide corresponding to

residues 170–181 of V2 binds directly to $\alpha_4\beta_7$. This same region of V2 overlaps the epitopes recognized by CH58 and CAP228-16H.

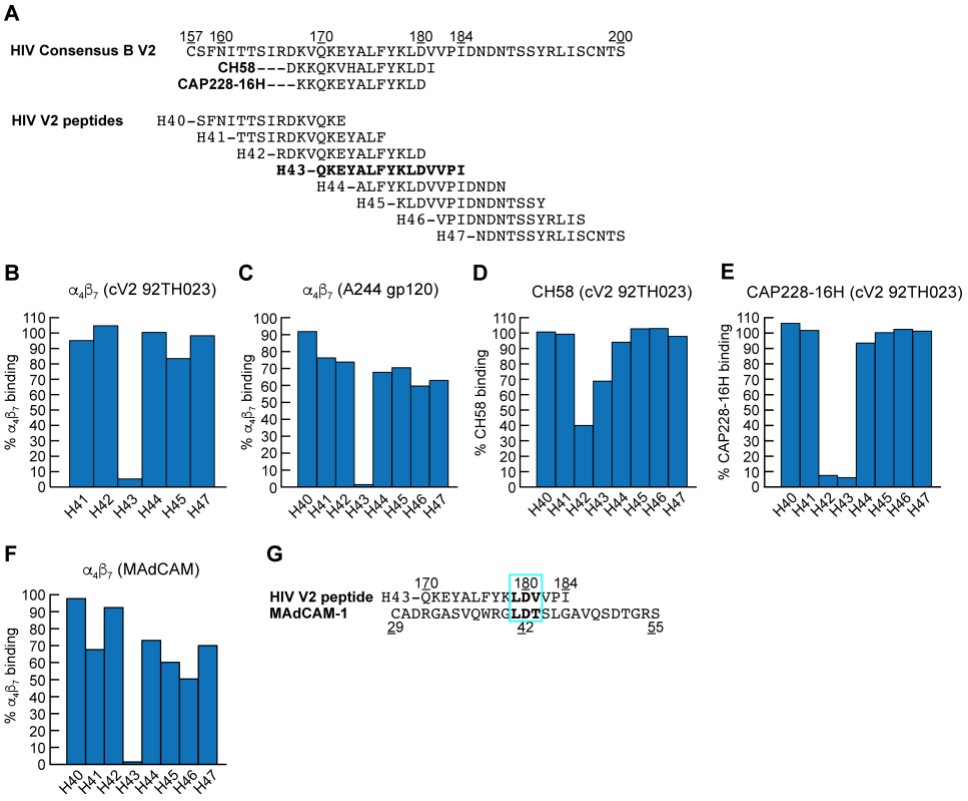


Figure 3.9. Inhibition of $\alpha_4\beta_7$ binding to cyclic V2, gp120, and MAdCAM by 15 aa linear peptides. A) Alignment of the V2 domains of HIV consensus B with the eight HIV consensus B 15 aa peptides (H40-H47) used in this study, and the regions of V2 recognized by mAbs CH58 and CAP228-16H. **B-C)** 15 aa peptide inhibition of $\alpha_4\beta_7$ binding to cV2 92TH023 and A244 gp120 as measured by SPR. Percent of $\alpha_4\beta_7$ binding relative to binding in the absence of peptide (defined as 100%) is shown. **D-E)** 15 aa peptide inhibition of V2 mAbs, CH58 and CAP228-16H, binding to cV2 92TH023. Percent mAb binding relative to binding in the absence of peptide is shown (y-axis). **F)** 15 aa peptide inhibition of $\alpha_4\beta_7$ binding to MAdCAM. Percent $\alpha_4\beta_7$ binding relative to binding in the absence of peptide is shown. **G)** Alignment of $\alpha_4\beta_7$ binding site in MAdCAM IgSF domain 1, and peptide H43. Conserved LDV/LDT binding motifs are boxed in blue.

3.5 Discussion

The lymphocyte gut homing receptor, integrin $\alpha_4\beta_7$, has increasingly been shown to play an important role in the transmission and pathogenesis of SIV and HIV [9, 24, 29,

30], both gut-tropic viral infections [1-4]. While $\alpha_4\beta_7$ is non-essential for $CD4^+$ T-cell infection by HIV *in vitro*, an antibody that antagonizes the interaction between $\alpha_4\beta_7$ and the V2 loop of gp120 can reduce infection, and can substantially affect disease outcome in an acute SIV transmission model [29]. When the same antibody was combined with ART, SIV infected animals were able to control viremia in a sustained way [30]. The underlying mechanism of viral control in this combination therapy study is not known. However, it was reported that all of the controlling animals, among other things, generated an anti-V2 specific antibody response that mapped to the $\alpha_4\beta_7$ epitope. It has been recently demonstrated in a cohort of women that $\alpha_4\beta_7^{high}$ $CD4^+$ T-cells are preferentially depleted from gut tissues as early as Fiebig I/II providing strong evidence that these cells serve as prime early targets following transmission [8]. This contributes to the connection between HIV's gut-tropic nature and $\alpha_4\beta_7$-mediated lymphocyte trafficking to the GALT. Weakly neutralizing Abs elicited in the RV144 Thailand HIV vaccine trial targeting the V2 loop have been implicated as a correlate of reduced risk of HIV acquisition [59-62]. Additionally, key residues in the V2 loop have been identified by molecular sieve analysis to be the target of vaccine elicited immune pressure [62]. These sieve sites fall around the binding epitope of $\alpha_4\beta_7$. Thus far, the mechanism whereby these weakly neutralizing V2p Abs might contribute to this effect has been unclear but may include Fc-mediated effector functions [39, 63, 64] , or an as yet identified function.

The Env that is expressed on the surface of an infected cell and subsequently incorporated into virions has been shown to be conformationally heterogeneous [65]. While the trimeric Env spike is associated with viral entry, a function of non-trimer associated Env has yet to be found. Immune evasion has been suggested as a potential role

for this seemingly aberrant Env. Env that is expressed on the surface of infected cells has been shown to have a second function: stabilizing what is known as a virological synapse (VS). Similar to an immunological synapse, a VS is a tight adhesive junction formed between an infected cell and an uninfected target cell, across which virus can be transferred efficiently. The oligomeric nature and/or maturity level of Env involved in the VS has yet to be characterized.

In the prefusion closed conformation of the Env trimer, the V2 adopts a β–barrel structure that is recognized by several bnAbs. The V2 can adopt an alternative conformation that is an unconstrained, helical conformation [55]. It is hypothesized that this helical conformation is more compatible with the CD4-bound, open conformation of gp120 that is somewhat freed from oligomeric constraints. The helical conformation of V2 is recognized by mAbs elicited by vaccination (CH58, CH59) and natural infection (CAP228-16H). The β–barrel conformation of the V2 in the prefusion closed trimer is incompatible with reactivity by these mAbs likely due to oligomeric constraints. Here, we show that the CH58-reactive helical conformation is present on the surface of infected primary cells. We also show that CH58-reactive Env is incorporated into virions. These findings demonstrate that the helical conformation of the V2 is present on the surface of infected primary cells as well as virions making the epitope a potential target by HIV vaccine candidates. The ratio between CH58-reactive Env and mature trimer Env on each virion is not known but our data suggests that a significant fraction of virions have at least one Env spike presenting the V2 in an unconstrained, helical conformation.

Because the lack of the helical conformation of V2 in the context of the Env trimer, it is hypothesized that the CH58-reactive, helical epitope is present on incompletely

processed or seemingly aberrant Env. In the pathway of post-translational processing, secreted and membrane-bound proteins are translated into the ER where initial post-translational modifications like the addition of high mannose glycans occur. After transport to the Golgi, subsequent rounds of processing, including modification of high mannose glycans into complex carbohydrate crowns, occurs. A fraction of Env that makes it to the surface of infected cells appears to lack the processing that takes places in the Golgi, and is therefore referred to as ER-Env. The glycosylation profile of Env has been shown to impact antigenic and functional characteristics of Env [66, 67]. Reports suggest that there is a connection between glycosylation and HIV transmission [68, 69]. Additionally, early-transmitting viruses are enriched in high-mannose carbohydrate [70]. Because ER-Env is also enriched in high-mannose carbohydrate, it is possible that there is a relationship between ER-Env and early-transmitting viruses. We show here that the CH58-reactive helical epitope is presented on Env that is processed in a Golgi-independent pathway. This suggests that the unconstrained, helical conformation of the V2 is associated with ER-Env and that ER-Env may play a role in the modestly protective anti-V2 response of the RV144 trial.

We have previously shown that the glycan profile of Env can not only impact the antigenicity of gp120, but that glycosylation can influence the reactivity of the V2 loop to $\alpha_4\beta_7$, in that removal of PNGSs increases binding of $\alpha_4\beta_7$ [25, 71]. Others have shown that trimming of glycans can increase the functional adhesion of $\alpha_4\beta_7$-expressing cells to the V2. We show here that, indeed, removal of glycans by PNGase F treatment reveals increased adhesion of $\alpha_4\beta_7$ to gp120s that without glycan trimming have low adhesion capacity in this assay. Taken together, this suggests that there may be steric influences by

glycans that can impact the structuring of the V2 that influence binding to $\alpha_4\beta_7$. However, it should be noted that not all Envs need to be deglycosylated to mediate $\alpha_4\beta_7$ adhesion.

Because of the connection between reduced glycosylation (ER-Env) and the capacity to bind $\alpha_4\beta_7$, we hypothesized that there may also be a connection between the unconstrained, helical conformation of the V2 and $\alpha_4\beta_7$-mediated adhesion. Here, we report that of the multiple conformations that the V2 region of gp120 can adopt, $\alpha_4\beta_7$ interacts with the V2 that is presented in a manner distinct from that of the closed Env trimer. A scaffolded V1V2 that presents an unconstrained form of the V2 mediates high levels of $\alpha_4\beta_7$-adhesion, whereas the same V1V2 fragment in an alternate scaffold presented as a β–barrel structure mediates only minimal adhesion. We show that a select group of V2 mAbs belonging to the V2p class inhibits the adhesion of $\alpha_4\beta_7$-expressing cells to the V2 loop of gp120. The V2p mAbs that inhibit adhesion (CH58, CH59 and CAP228-16H) recognize a helical or coiled conformation of the V2. Our finding that mAbs CH58 and CH59, which were derived from RV144 vaccine recipients, blocks V2-mediated adhesion to $\alpha_4\beta_7$ raises the possibility that antibody activities distinct from both neutralization and Fc-mediated effector functions might contribute to the efficacy of the RV144 HIV vaccine. Supporting this concept is that mAb NCI09, which was derived from a macaque administered an SIV_{mac251}-based vaccine designed to mimic the RV144 vaccine [72], also blocks V2-mediated adhesion to $\alpha_4\beta_7$ [47]. As in RV144, reduced risk of infection in animals administered this vaccine was correlated with weakly neutralizing anti-V2 antibodies [72].

We also show that the way in which $\alpha_4\beta_7$ interacts with the V2 region of gp120 shares key features with the interaction between $\alpha_4\beta_7$ and its natural ligand, MAdCAM. This apparent mimicry may have important implications in HIV pathogenesis, particularly

in regard to the role of the gut in the development of HIV disease. It may also impact anti-V2 loop immune responses in both infected and vaccinated subjects. One consequence of this mimicry is that antagonists developed to treat IBD interfere with V2- $\alpha_4\beta_7$ interactions.

The V2 domain of HIV gp120 varies in both length and sequence identity. Yet we find that V2s from three subtypes of HIV retain the capacity to bind to $\alpha_4\beta_7$, suggesting that this interaction is a general property across HIV subtypes. Other studies failed to detect a specific interaction between $\alpha_4\beta_7$ and gp120 [51, 73]. An explanation for this discrepancy likely reflects two variables. First, as our group and others have shown, the addition of glycans can reduce the interaction between recombinant gp120 with $\alpha_4\beta_7$ [25, 46]. It is likely that excess amounts of complex carbohydrate and sialic acid moieties that are added to gp120s expressed in cell lines contribute to this inhibitory effect. Of note, we demonstrated that highly purified (> 95%) cyclic V2 loop peptides that lack glycans bind $\alpha_4\beta_7$ with high affinity in both SPR-based binding assays and in a cell-based adhesion assay. Removal of glycans in order to observe $\alpha_4\beta_7$ reactivity is not an absolute requirement for binding insofar as we were able to capture virions derived from primary cells with $\alpha_4\beta_7$ coated nanoparticles. The second variable that may influence the sensitivity of $\alpha_4\beta_7$ binding assays involves the expression level, and state of $\alpha_4\beta_7$ on cell surfaces. Many integrins rely on complex avidity effects and clustering in order to engage ligands. It is likely that the surface density of $\alpha_4\beta_7$ plays a key role in its interaction with gp120.

The specific affinity of $\alpha_4\beta_7$ for gp120 is comparable to that of MAdCAM. Among the integrins expressed in humans, $\alpha_4\beta_7$ is distinct in its ability to mediate both lymphocyte rolling, and firm adhesion, which reflects the highly specialized nature of MAdCAM-$\alpha_4\beta_7$ interactions. Integrin $\alpha_4\beta_7$ activity is modulated by dynamic changes in the overall structure

of the receptor. It is notable that the V2 region of gp120, despite its variable sequence, is able to mimic the binding of $\alpha_4\beta_7$ to MAdCAM. Two pieces of evidence support V2 mimicry of MAdCAM. First, MAdCAM utilizes divalent cations, which coordinate with the D^{42} in MAdCAM and the MIDAS of β_7. The V2 depends on divalent cations for $\alpha_4\beta_7$ binding in the same way that MAdCAM does. Importantly, the conformational state (inactive, intermediate or active) of $\alpha_4\beta_7$ is responsive to both intracellular and external cues that are linked to cellular signals generated during inflammatory responses [74, 75]. The ability of V2 to discriminate between different forms of $\alpha_4\beta_7$ provides a mechanism to distinguish between different subsets of lymphocytes, including those with high potential to home to GALT. Given the propensity of HIV to replicate in GALT, it is tempting to link the preferential infection and depletion of $\alpha_4\beta_7^{high}$ $CD4^+$ T cells in the very early stages of infection [8, 27, 76], with V2-$\alpha_4\beta_7$ interactions. However, such a link has not yet been established.

The second line of evidence that supports the proposition that V2 mimics the binding characteristics of MAdCAM comes from our demonstration that $\alpha_4\beta_7$ antagonists that were developed to block binding to MAdCAM, also block binding to V2. Because of the increase in incidence and prevalence of IBD [77], considerable efforts have been taken to develop effective treatments, including drugs that target $\alpha_4\beta_7$. Detailed structural characterizations of both MAdCAM and $\alpha_4\beta_7$ have been employed in the rational design of small molecule LDV mimetics [78]. These mimetics bind with precision to the MAdCAM binding site on $\alpha_4\beta_7$, which lies within a deep groove formed by the α_4-β_7 interface [14]. By showing that one of these mimetics compete with V2, we conclude that the aliphatic amino acid-Asp motif conserved in HIV fit into this groove and engage $\alpha_4\beta_7$ in a way that,

at least partially, mimics the L^{41}-D^{42} encoded in the MAdCAM CC' loop of IgSF domain 1 [79, 80]. These results suggest that the carboxy-terminus of V2 and this IgSF domain 1 CC' loop can adopt similar conformations. Evidence for the conserved nature of this structure comes from the observation that one SIV V2 mAb, ITS03, whose epitope maps close to the $\alpha_4\beta_7$ binding site, blocks $\alpha_4\beta_7$ adhesion to V2 and also cross-reacts with an HIV subtype A/E V2 [47]. This raises the intriguing possibility that, with additional screening, one might identify a V2 mAb that cross-reacts with MAdCAM. Indeed, other regions of the HIV envelope have been shown to mimic "self" epitopes [81].

How weakly neutralizing antibodies that block V2-mediated adhesion to $\alpha_4\beta_7$ might contribute to reduced risk of infection is unknown. We previously reported that the V2 region of gp120 can deliver cellular signals through $\alpha_4\beta_7$ [9]. In this regard, integrins including $\alpha_4\beta_7$ are key components in integrin associated complexes that are able to modulate biochemical pathways and reorganize both cell-surface receptors and the actin-cytoskeleton [74, 82]. In addition, signaling through $\alpha_4\beta_7$ on $CD4^+$ T cells can impact cell activation, proliferation and apoptosis [83, 84]. We recently reported that MAdCAM costimulation through $\alpha_4\beta_7$ supports HIV replication in $\alpha_4\beta_7^{high}$ $CD4^+$ T cells [85]. Whether V2 signaling through $\alpha_4\beta_7$ can similarly support HIV replication requires additional investigation. Such information will help determine whether this type of signaling could facilitate HIV transmission/replication and whether antibodies that interfere with this signal can reduce the risk of infection.

The way in which the V2 region of gp120 engages $\alpha_4\beta_7$ shares key features with the way that MAdCAM, a receptor expressed primarily in gut tissues, engages $\alpha_4\beta_7$. One consequence of this apparent mimicry is that antagonists developed to treat IBD by

interfering with the interaction between MAdCAM and $\alpha_4\beta_7$ also interfere with V2-$\alpha_4\beta_7$ interactions. These findings suggest that V2 mimicry of MAdCAM in its interactions with $\alpha_4\beta_7$ may influence early events in HIV infection and replication in GALT. Thus, further investigation of this interaction and its likely role in early HIV infection will aid in our efforts to develop effective prevention and therapeutic strategies.

The nature of the epitope in V2 that engages $\alpha_4\beta_7$ appears to involve a structure that is not present in a recombinant trimer designed to mimic the closed trimeric Env spike. Antibodies that target this epitope and block V2-$\alpha_4\beta_7$ interactions are not themselves broadly neutralizing, although the structure that they recognize is conserved across the HIV clades. Because the weakly neutralizing Abs elicited in RV144 inhibit the interaction, it is possible that a connection between the V2-$\alpha_4\beta_7$ interaction and the observed protection in RV144 exists. As yet, there is no data to suggest that disrupting the V2-$\alpha_4\beta_7$ interaction contributed to RV144 vaccine elicited protection. However, this and other data suggest that it is possible that protection could be achieved by eliciting a conformationally specific antibody response. Inducing conformationally specific antibody responses to the V2 loop has already been accomplished using scaffolded V1V2 peptides in rabbits [86, 87]. However, much work is needed to determine if such an antibody response inhibits $\alpha_4\beta_7$ binding or provides protection in the context of HIV transmission.

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> Wibmer CK, Richardson SI, Yolitz J, Cicala C, Arthos J, Moore PL, Morris L. (2018). *A common helical conformation of V1V2 exposes the* a*4*b*7 binding site on intact HIV-1 virions.* Nat Commun. 9(1): p. 4489.

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Chapter 4:
CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Rationale for Studying Genetic Signatures of HIV-1 Transmission

Development of an HIV vaccine that elicits protective immunity has proven difficult despite findings that mucosal transmission of HIV is inefficient. One such difficulty in development efforts is that such a vaccine will need to elicit immune responses to a broad spectrum of diverse circulating HIV isolates. To combat this obstacle, researchers have sought to characterize conserved signatures of viral isolates with greater transmission potential or fitness. If viruses isolated immediately after transmission or very early in infection have a constrained pattern of sequence diversity, designing a vaccine to target such signatures would be beneficial in further reducing the efficiency of HIV transmission.

It has long been understood that high genetic diversity of HIV exists in a single HIV infected individual during the chronic phase of infection [1]. This is due to a high mutation rate of HIV replication machinery and the eventual selective pressure by the host immune system. However, sequence diversity is greatly reduced during transmission [2-5]. Very often one or very few viral isolates establish a productive infection in a recipient. This phenomenon is referred to as the genetic bottleneck of HIV transmission. It is reported that dissemination of a single variant occurs in 80% of heterosexual, 75% of MSM, and approximately 70% of mother-to-child transmission events [6-12]. The rate of infection by a single variant in transmission events of injection drug users is not entirely clear. Many key questions about the selective pressures and events that surround mucosal transmission and the genetic bottleneck that occurs, remain unanswered. Identifying characteristics that govern HIV transmission and infection are essential for the development of effective, targeted prevention strategies. Ideal vaccine candidates elicit a response that blocks

productive infection by targeting viral proteins in a way that disrupts initial infection or significantly reduces transmission fitness. In this way, identifying common, conserved characteristics of transmitting viral isolates may lead to targets of prevention strategies that could impede transmission.

In these efforts, several groups have compared HIV Env sequences of isolates associated with the early stages of infection to isolates associated with the chronic phase of infection. The first of such studies identified that transmitting viral isolates encoded compact, glycan-restricted Env proteins [13]. This genetic signature involved a reduced number of PNGSs and shorter variable loops of gp120 in which many of these PNGSs are located. Other studies showed that specific amino acid residues and motifs were the subject of selective pressure: selection away from H^{12} in the signal peptide of Env during the chronic phase of HIV/SIV/SHIV infection, selection for $I^{33}A^{55}K^{57}N^{88}$ at the time of SIV transmission, and selection for $A^{55}D^{57}N^{88}$ at the time of HIV and SHIV transmission [14-16]. These studies show that conserved signatures of HIV, SIV and SHIV transmission are under strong selective pressure at specific residues, and therefore, transmission is not a stochastic event for the diverse swarm of viruses replicating in a donor [17].

The field's efforts to develop a robustly protective vaccine have proven generally unsuccessful. To date, there have been over 220 phase I/II/III HIV vaccine trials, only six of which have studied efficacy explicitly [18, 19]. Of these efficacy trials, only one has indicated a moderate signal of protection: the RV144 Thailand HIV vaccine trial [20]. Even with only 31.2% estimated vaccine efficacy, much has been done to determine any potential mechanisms associated with reduced risk of HIV acquisition in this trial. These

efforts are paramount for identifying correlates of protection in our efforts to optimize a protective HIV vaccine.

However, the identification of immune correlates of reduced acquisition from the RV144 trial suggest that an achievable mechanism of protection may not be the vaccine-elicited immune response that researchers originally intended to induce with this vaccine. As described previously in this thesis, only weakly neutralizing antibody responses were elicited in RV144 vaccine recipients. Additionally, antibodies targeting the CD4 binding site of gp120 or other "super site" targets of bnAbs were not correlated with reduced risk of infection. While neutralizing antibodies may have the capacity to protect non-human primates (NHPs) from SIV infection, these findings support the notion that improving the alternative mechanism of protection that was involved in achieving moderate protection in RV144 is an attractive approach. The search for an alternate mechanism is vigorously underway.

Trials involving human subjects where sequence data can be collected from matched acute/early isolates and donor isolates are unfortunately sparse. By definition, transmitting viral isolates possess characteristics necessary for viral transmission. However, without donor sequence data, it is difficult to form conclusions about the existence of selective pressure. This precludes the ability to sufficiently and accurately detect genetic signatures of transmission in a statistical way. This highlights the importance of ongoing discordant couple cohort studies where donor sequence data is available. However, these protocols generally, and ethically, provide education and materials that reduce the risk of transmission, and therefore reduce the likelihood of a matched transmission event from occurring. Overcoming this obstacle may prove futile. While not

ideal, one potential solution to the lack of donor matched sequence data is to conduct SIV transmission studies with more genetically diverse stock viruses which could provide more statistically robust analysis of genetic signatures.

In *chapter 2*, we describe characteristics of gp120 that are influenced by one such genetic signature of transmission that was identified in HIV subtype B isolates and later confirmed in other HIV sequence cohorts, as well as SIV and SHIV transmission studies [16]. We show that an overrepresentation of basic amino acids in the SP of HIV Env biases the glycosylation pattern of gp120 in a significant way. Several reports have shown that the glycosylation pattern of gp120 plays a major role in the structure and function of gp120. We find that significant changes to the glycan profile can be modulated by subtle, selectable changes in the SP sequence. This modulation does not rely on mature coding sequence mutations but rather variation in a portion of the propeptide that is not present in the mature protein. We show that these changes to the glycan profile dramatically impact gp120 binding by DC-SIGN, a C-type lectin receptor on DCs that is believed to play a role early in transmission. We hypothesize that these changes likely impact reactivity to other receptors that have gp120 glycan or structural requirements for their binding, potentially integrin $\alpha_4\beta_7$.

We also report that the genetic signature of transmission in the SP of Env also impacts the antigenicity of gp120. We report that by swapping the SP of a recombinant gp120 from one bearing the signature to one lacking the signature, the resulting gp120 adopts are more closed structure. This is in agreement with other data suggesting that the virus is selecting for a more guarded gp120 during the chronic phase of infection. Escape from immune pressure requires HIV to shield receptor/co-receptor binding sites and other

neutralizing epitopes of Env. This signature may be highlighting a mechanism by which the virus can modulate the shielding of these sites. The biological relevance of such a mechanism is significant.

In the effort to develop vaccine immunogens, the SP of Env is often neglected. Many immunogens are produced with a heterologous SP from another human or viral protein. This is done with good intentions: to produce large amounts of recombinant protein. However, there may be discrepancies in key features of these recombinant immunogens and the natural protein of interest. Our data shows that the SP alone can alter the structure of recombinant proteins that contain the same mature coding sequence. This suggests that the resulting structure of these recombinant immunogens with heterologous SPs is likely different than the intended antigenicity. This likely has major impacts on the immunogenicity of the recombinant antigens.

However, the fact that subtle changes in the SP can modulate the antigenicity of immunogens highlights a potential tool in vaccine design. By making changes to the SP of recombinant immunogens, modifications to the glycan profile and the antigenicity can be accomplished while not altering the mature coding sequence. This may allow for the use SP variants to modulate the antigenicity of conserved primary amino acid sequences. Whether the SP can bias the immune response to an immunogen is worthy of intense research focus.

The vaccine efficacy observed in the RV144 Thailand HIV vaccine trial was not great enough to meet the epidemic's needs. The field has, however, gained powerful insights from RV144 into potential mechanisms of protection and correlates of immunity. Building upon these findings has proven complex. With additional knowledge gained from

studies of genetic signatures of transmission, previously underappreciated links between transmission and Env structure can be explored. This thesis research aimed to make connections between genetic signatures of transmission and functional aspects of Env that are potential targets of the RV144-elicited immune response.

4.2 A Role for $\alpha_4\beta_7$ in HIV Transmission and Pathogenesis

It has been shown that expression of $\alpha_4\beta_7$ on $CD4^+$ T-cells in linked with cellular activation. These $\alpha_4\beta_7^+CD4^+$ T-cells also express Ki67, CD69 and CCR5 marking them as ideally activated target cells for HIV infection. Additionally, $\alpha_4\beta_7$ colocalizes with CD4 and CCR5 on the cell surface [21]. The coupling of these receptors reasonably implies that HIV may utilize $\alpha_4\beta_7$ as an attachment factor and/or marker of highly activated target cells that support viral replication. In the context of the genital mucosa, a majority of the $CD4^+$ T-cells are not activated and therefore are not ideal targets for productive infection. One could consider the possibility that evolutionary pressure has adapted HIV to interact with $\alpha_4\beta_7$ as a way to increase the likelihood of a productive infection. In this way, the affinity for $\alpha_4\beta_7$ indicates an opportunity for HIV to increase its transmission fitness.

One way of studying the role of $\alpha_4\beta_7$ in transmission is to antagonize the interaction. Passive infusion of a primatized anti-$\alpha_4\beta_7$ mAb did not protect rhesus macaques from a single high dose challenge of pathogenic SIV_{mac239} [22]. However, it did protect the GALT from high levels of viral replication. A more physiologically relevant way to evaluate the role of $\alpha_4\beta_7$ in SIV transmission would be to conduct a similar experiment with a viral inoculum more reflective of natural infection. This was done by infusing the same anti-$\alpha_4\beta_7$ mAb and challenging the animals weekly with a low dose of pathogenic SIV_{mac251} intravaginally [23]. The mAb infusion significantly delayed infection for 6/12 animals

while 10/12 control animals were rapidly infected. The anti-$\alpha_4\beta_7$ mAb protected the remaining 6/12 rhesus macaques from infection. Of the treated animals that became infected, there was significant protection of the $CD4^+$ T-cell population in the GALT. This shows that targeting $\alpha_4\beta_7$ reduces mucosal transmission of SIV in macaques.

Additionally, it has been shown that $\alpha_4\beta_7$ may play a role in the context of acute SIV infection. Infusions of an anti-$\alpha_4\beta_7$ mAb given to *rhesus macaques* infected with pathogenic SIV_{mac239} but suppressed with daily ART lead to sustained virologic control even after all therapies were terminated [24]. All of the control animals had viral rebound after termination of ART while the mAb-treated animals either had blips in viremia or had no viral rebound at all. The treated animals showed significant protection of the GALT $CD4^+$ T-cell population. Additionally, 6/6 of the treated animals developed a unique anti-V2 immune response that only 3/7 control animals developed. This anti-V2 immune response is similar to that observed in RV144 vaccine recipients and correlates with reduced risk of HIV and SIV acquisition [25-28]. This data suggests that combination therapy of ART and anti-$\alpha_4\beta_7$ mAb allows macaques to effectively control viremia without the need for further therapy. It also raises questions as to the mechanism(s) by which targeting $\alpha_4\beta_7$ can block transmission and reconstitute an immune response that controls viremia.

Although the link between $\alpha_4\beta_7$ expression and highly activated cells that are ideal for viral replication seems to provide sufficient basis for HIV to evolve the capacity to interact with $\alpha_4\beta_7$, the integrin has several other functions that may provide further selective advantage to develop such an interaction. One such function is that $\alpha_4\beta_7$ also holds the capacity to serve as a costimulatory receptor [29, 30]. Our group recently provided

evidence that MAdCAM stimulation of naïve and memory $CD4^+$ T-cells through $\alpha_4\beta_7$ leads to activation and proliferation that supports viral replication [31]. This MAdCAM signaling through $\alpha_4\beta_7$ is blocked by the anti-$\alpha_4\beta_7$ mAb that prevents mucosal transmission of SIV in the SIV transmission study described above. Considering the mimicry of MAdCAM by the V2 loop of gp120 presented in *Chapter 3* and our work showing that MAdCAM co-stimulation leads to activation and proliferation that supports viral replication, it is possible that the V2 loop can also provide this costimulatory signal. Studies aiming to address this possibility are currently underway. This is one potential mechanism in which targeting $\alpha_4\beta_7$ reduces SIV transmission and pathogenesis.

While the mechanism by which HIV and SIV utilize $\alpha_4\beta_7$ in the context of transmission and pathogenesis is still under investigation, the importance of $\alpha_4\beta_7$ in HIV acquisition and disease progression has recently been studied. Much of the previous work described has been done in an *in vitro* setting or through the use of animal models. However, in a prospective natural history study of a cohort of at-risk women, pre-HIV infection frequencies of $\alpha_4\beta_7^+CD4^+$ T-cells were shown to correlate with increased rates of HIV acquisition [32]. This association was stronger when infection was caused by an isolate containing V2 motifs with a preference for $\alpha_4\beta_7$ binding. Additionally, pre-infection frequencies of $\alpha_4\beta_7^+CD4^+$ T-cells predicted a higher set-point viral load and an increased rate of $CD4^+$ T-cell decline. This data shows that $\alpha_4\beta_7^+CD4^+$ T-cells play a role in HIV transmission and disease progression. In the colon of these individuals, $\alpha_4\beta_7^+CD4^+$ T-cells were rapidly depleted within days of infection, and were not recovered even after 24 months of ART. In contrast, $CCR5^+CD4^+$ T-cells were not significantly depleted in the colon until Fiebig stage III, and the frequency of these cells in the gut returned to near

baseline levels after 6-24 months of ART. These data show directly that $\alpha_4\beta_7^+CD4^+$ T-cells are preferentially infected and irreversibly depleted during acute HIV infection and that the pre-infection frequency of these cells correlates with risk of HIV acquisition and disease progression. This data confirms our initial report that HIV preferentially infects and depletes $\alpha_4\beta_7^+CD4^+$ T-cells *in vitro*. It also underscores the implicit connection between the gut homing receptor integrin $\alpha_4\beta_7$ and the gut-tropic nature of HIV infection and pathogenesis.

In this thesis, we present data highlighting the mimicry of MAdCAM in the way the V2 loop of gp120 interacts with $\alpha_4\beta_7$. Previous work has shown that the interaction between the V2 and $\alpha_4\beta_7$ is mediated by a conserved LDV/I motif in the V2 that is similar to that of the LDT motif in MAdCAM involved in $\alpha_4\beta_7$ binding [33]. Our group has also shown that the functional capacity of $\alpha_4\beta_7$ adhesion is cation-dependent for both MAdCAM and V2 binding [34]. Additionally, we show that inhibitors of the MAdCAM-$\alpha_4\beta_7$ interaction also block the adhesion of V2 to $\alpha_4\beta_7$. This highlights the potential for therapeutics designed to disrupt the MAdCAM-$\alpha_4\beta_7$ interaction to also interfere in HIV-$\alpha_4\beta_7$ interactions. Further investigation into MAdCAM antagonists as potential inhibitors of HIV-$\alpha_4\beta_7$ interactions are needed to better our understanding of this mimicry.

We also show that a select class of V2 mAbs can interfere with the interaction between $\alpha_4\beta_7$ and the V2 loop. These antibodies have been isolated from RV144 vaccine recipients (mAbs CH58 and CH59) and from natural infection (mAb CAP228-16H). These antibodies recognize an epitope that overlaps with not only the $\alpha_4\beta_7$ interaction binding sites ($Q^{170}K^{171}E^{172}$ and $L^{179}D^{180}V^{181}$) but also the RV144 sieve sites (K^{169} and I^{181}). This class of antibodies recognizes a conformation of the V2 that is absent from the SOSIP

stabilized trimer. In the trimer, the V2 adopts a constrained β-barrel or Greek key conformation. However, outside the context of the stabilized trimer, the V2 can adopt an alternative conformation that is helical or coiled. The mAbs that block $\alpha_4\beta_7$ adhesion to the V2 loop recognize a conserved helical conformation of V2. This data suggests that the way in which the V2 loop is presented plays a significant role its capacity to bind $\alpha_4\beta_7$.

Our data shows that the way in which the V2 is presented in the artificially stabilized trimer is not compatible with $\alpha_4\beta_7$ binding. However, we show that $\alpha_4\beta_7$-reactive Env is incorporated into virions in that magnetic nanoparticles coated with $\alpha_4\beta_7$ are capable of capturing a significant fraction of virus from infected supernatants. We show that mAb CH58, which inhibits $\alpha_4\beta_7$-V2 interactions, is capable of capturing a significant fraction of virions as well. This data shows that the V2 is presented in a way that is $\alpha_4\beta_7$ reactive on the surface of virions.

The connection between the weakly-neutralizing Abs elicited by RV144 and their ability to inhibit $\alpha_4\beta_7$ adhesion, suggests a potential connection between the functional targets of RV144 mAbs and the observed protection. There exists no evidence that disrupting the $\alpha_4\beta_7$ interaction would contribute to protection in the context of a vaccine, however data from our group and others suggests that achieving such a response may require eliciting antibodies to a specific, alternative conformation of the V2. The proof of concept for generating conformationally specific immune responses has been done. Immunization of rabbits with alternative V1V2 scaffolded immunogens induced distinct conformation-specific Ab responses [35, 36]. Whether these Ab responses inhibit $\alpha_4\beta_7$ binding or provide protection have yet to be determined. Much work is needed to confirm this intriguing hypothesis.

4.3 Implications for Post-Translational Processing in Vaccine Immunogen Design

Post-translational modifications can directly and indirectly impact the flexibility and availability of gp120 epitopes. This is accomplished by the direct and indirect steric influence of glycans on adjacent residues or motifs in close proximity after folding. In vaccine design, the structure in which the immunogen adopts is critical to the specificity of the immune response elicited. Here we show that a shift in glycan profile can impact the antigenicity of mature sequence-identical recombinant proteins. By shifting the glycan profile to contain a larger proportion of high mannose glycans, we shift the open-ness of the gp120 protein as measured by reactivity to CD4i mAbs. This was accomplished through the use of natural variation in the SP of Env. We also show that the SP can influence the neutralization profile of viruses, particularly by anti-V2 mAbs. This suggests that the glycosylation profile altered by natural variation in the SP can greatly impact the reactivity of neutralizing mAbs to gp120, an important finding in the context of vaccine induced immunity.

Much work has been done using recombinant gp120 proteins as immunogens in HIV vaccine designs. However, in nearly all of these studies, the recombinant protein is generated from a construct that replaces the natural SP of the HIV Env with that of a heterologous viral SP or one from other proteins. In fact, the RV144 vaccine trail used the A244 gp120 sequence but swapped its natural SP for that of HSV gD protein. The use of heterologous SPs for vaccine immunogen production is done, quite often, for the generation of large quantities of protein. Unfortunately, as we have shown, the SP plays a major role in the antigenicity of recombinant gp120s through altered PTM. Immunogenicity studies in mice looking at the impact of SP-induced glycan changes on

the immune response are currently underway. Initial observations suggest that the SP can influence the antibody response. In these preliminary studies, the magnitude of response against a cyclic V2 peptide does not appear to differ. However, the reactivity of sera to conformationally biased V1V2 scaffolds does appear to vary. These findings, while extremely preliminary, suggest that the SP can impact the immunogenicity of gp120 proteins. Further work is needed to identify the differences in antibody responses elicited by SP chimera immunogens. This work could greatly aid in the design of vaccines by adding another tool by which immunogenicity can be modulated.

Most immunization studies have failed to induce broadly neutralizing Abs or Abs that protect from infection. It was surprising to find that the vaccine elicited Ab response from RV144 was weakly-neutralizing in the standard neutralization assay. The standard neutralization assay protocol involves an hour-long incubation of Abs with pseudoviruses before testing neutralization. It is noteworthy that Hioe and colleagues show that a longer incubation time (24 h) significantly increases the neutralization capacity of mAbs CH58 and CH59, particularly to the 92TH023 isolate [37]. The explanation for this observation is not currently known. However, one possibility is that these mAbs may have binding kinetics (i.e. on/off rates) that require longer exposure to the V2 loop. Additionally, it is possible that the V2 conformation that they recognize, is not often presented. It may be that gp120, which samples multiple conformations, does not readily adopt the helical conformation recognized by these mAbs. It is likely that glycosylation impacts the rate in which gp120 adopts alternate conformations and therefore access by broadly or weakly neutralizing antibodies.

In efforts to induce neutralizing, and theoretically protective, mAbs, much has been done using the stabilized Env trimer as an immunogen. Our group and others have provided ample evidence that the epitope of gp120 in which $\alpha_4\beta_7$ engages is discontinuous and requires flexibility in the way in which it is presented. Because the lack of bnAbs elicited in the moderately protective RV144 trial, it is possible that an alternative approach that does not rely on a trimer-targeting bnAb response may provide sufficient protection. While this suggestion is speculative, a vaccine immunogen intended to induce a conformationally-specific Ab response may provide such an alternative mechanism of protection [35, 36]. It is noteworthy that the Ab response elicited in RV144 appears to recognize a conformation-specific epitope that is absent from the stabilized trimer. In fact, the RV144 elicited immune response targeted a conserved helical epitope that is not presented on fully formed recombinant trimers. We show here that the form of Env recognized by CH58 is incompletely processed ER-Env. We also show that mAb CH58 is capable of capturing a significant fraction of virions from infected cell supernatants. This shows that while these Abs may not inhibit infection in the field's current neutralization assay, they still have biological relevance and are associated with reduced risk of infection. One potential hypothesis for this mystery is that the alternate form of ER-Env that these Abs are recognizing may play an underappreciated role in HIV transmission that when blocked may provide some level of protection. Efforts to better understand the role of ER-Env in infection and transmission are needed. Additional studies are needed to determine the immunogenicity of such differently processed forms of Env.

As described in this thesis, CH58-like mAbs recognize an incompletely processed form of Env on the surface of infected cells and virions. Crystal structure data shows that

these mAbs recognize a helical conformation of the V2 that is presented in an unconstrained manner. These CH58-like mAbs inhibit $\alpha_4\beta_7$ binding and we show that $\alpha_4\beta_7$ itself is capable of capturing virions. Together this data suggests a connection between ER-Env and $\alpha_4\beta_7$ binding that is relevant to virions and infected cells. Because CH58 is associated with the RV144 vaccine trial that exhibited moderate protection, it is alluring to hypothesize that that $\alpha_4\beta_7$-reactive ER-Env may play a role in transmission and is therefore worthy of incorporation into vaccine design. However, we do not know the precise role $\alpha_4\beta_7$-reactive ER-Env in HIV infection, transmission and pathogenesis nor do we know how differences in incompletely processed Env impact immunogenicity. Much work is needed in the area of $\alpha_4\beta_7$-reactive ER-Env in order to answer these questions.

4.4 Summary and Future Directions

The main goal of this thesis was to study the connections between genetic signatures of HIV transmission and critical post-translational processing of HIV Env. To answer important questions about these topics we incorporated numerous studies of Env spanning sequence variation, glycosylation, structure and function. The initial aim of this work was to connect a genetic signature located in the SP of HIV Env to variations in Env glycosylation. While others have shown that the SP can impact characteristics of Env maturation, these previous studies have made use of heterologous SPs from human and insect proteins. No other studies have shown that natural variation found in the SP of Env associated with transmission could directly lead to an altered glycosylation profile of gp120. We show that not only can natural SP variation influence the glycan profile of gp120, but that this change in glycosylation impacts reactivity with the receptor DC-SIGN. In addition, we showed that the SP alone could impact the antigenicity of gp120 in a major

way. Future work should include looking into the impact of HIV Env SP on binding kinetics of other transmission-relevant receptors such as $\alpha_4\beta_7$.

We have outlined several reasons as to the significance of the influence of the Env SP. Biologically, this data suggests that the SP has major influence over the post-translational processing pathway in which transmembrane and secreted proteins traverse. In the context of HIV, this suggests that the virus has evolved a mechanism by which it can easily modify the glycan shield in an apparent attempt to mask neutralizing epitopes or modulate reactivity to transmission-relevant receptors. Selection by the virus for a direct mutation in the coding sequence of the mature protein has a potentially high risk of failure and/or structural sacrifice. Many of these mutations will be fatal to the virus; a high evolutionary cost. However, the evolutionary cost for a mutation in a region not present in the mature protein is significantly lower. In fact, if this mutation slightly modifies Env without sacrificing the already proven functionality of the mature protein, the mutation would be an evolutionary benefit. That the virus has adapted a method of shifting the glycan profile to shield from building immune pressure or modify receptor reactivity without altering the coding sequence of mature protein is remarkable. This highlights the complexity by which the virus is able to evade clearance by the immune system and maintain infectivity.

The identification of the SP signature was made without bias towards characteristics that influence neutralizing epitopes. Remarkably, we find that the SP of Env can impact the antigenicity of gp120 through altered glycosylation. We also find that natural variation in the SP can alter the neutralization profile of SP chimera viruses, particularly by anti-V2 mAbs. Future studies of the impact of natural variation in the SP

on neutralization are needed and will provide a better understanding of the way in which HIV can modulate key features of Env. Additionally, the SP chimera viruses that we have generated have provided us a tool by which to study the impact of the SP on the expression of Env. One could presume that if the SP can impact gp120 antigenicity, the SP may also impact the proportion of ER-Env versus fully-formed trimer Env that is expressed on the surface of infected cells or incorporated into virions. Our SP chimera viruses may aid in furthering our understanding of the connection between Env characteristics associated with transmission and the SP signature.

In our efforts to define how post-translational modification impacts the structure of gp120, we found that mAbs isolated from RV144 (mAbs CH58) recognize Env that is processed in a Golgi-independent pathway. How an antibody response against incompletely processed Env could mediate protection is not immediately apparent. However, we show that CH58-reactive Env is present on the surface of infected cells and the surface of virions from infectious supernatants. While our data shows that a significant fraction of virions from viral supernatants contain at least one CH58-reactive Env, the significance of ER-Env in the context of cell-free and cell-to-cell spread has not been identified. It is currently unknow what proportion of Env on the surface of virions is reactive with CH58-like mAbs (i.e. ER-Env versus fully formed trimers). Additionally, it is not known what fraction of Env on the surface of an infected cell is reactive with CH58-like mAbs. Is the proportion of ER-Env similar between infected cells and virions? Does the ratio change during the course of a single cell's infection? Can the SP influence this ratio? Does the ratio change during the course of disease progression? These questions are important in our understanding of the significance of the helical V2 conformation and what

controls its presentation. More investigation is needed into the ratio of CH58-reactive Env on the surface of infected cells and virions, and the role that the unconstrained presentation of the V2 plays in HIV transmission.

The answers to the questions above will provide a better understanding of the abundance of ER-Env in the context of infected cells and virions. However, the functional role of ER-Env is still not well characterized. It has been proposed that ER-Env and other aberrant Env expression is a mechanism by which HIV can distract the adaptive immune response. It is possible that HIV has developed the ability to express ER-Env as a way to increase exposure of non-neutralizing or immunodominant epitopes, thereby distracting the immune response from "unwanted", neutralizing epitopes of the trimer. However, we believe that ER-Env plays a more functional role in the spread of HIV. Our group hypothesizes that the unconstrained conformation of the V2 that is precluded from the stabilized trimer is the conformation that mediates adhesion of $\alpha_4\beta_7$. We have previously shown that reduced glycosylation and removal of PNGSs increases binding of gp120 to $\alpha_4\beta_7$, suggesting a connection between reduced PTMs and $\alpha_4\beta_7$ reactivity [38]. Data presented here and by others suggests that ER-Env is capable of interacting with $\alpha_4\beta_7$. This work suggests a further connection between PTM and glycosylation and the unconstrained structure in which $\alpha_4\beta_7$ engages. We show that mAb CH58, which recognizes an unconstrained helical conformation and inhibits adhesion of $\alpha_4\beta_7$ to the V2 loop, is reactive to incompletely processed Env. The mAb CH58 and $\alpha_4\beta_7$ are capable of capturing a significant fraction of virions from viral supernatants. These data show that the unconstrained conformation of the V2 loop that engages $\alpha_4\beta_7$ is present on the surface of virions. The physiologically relevant function of ER-Env is not currently known, although

it is unlikely that this form of Env is directly involved in membrane fusion. It is possible that one potential role of ER-Env is engagement with $\alpha_4\beta_7$. Further studies are needed to confirm this hypothesis.

It is known that $\alpha_4\beta_7^+CD4^+$ T-cells play a critical role in HIV transmission and pathogenesis. However, our understanding of the precise way in which HIV utilizes $\alpha_4\beta_7$ is incomplete. Does ER-Env on virions bind $\alpha_4\beta_7$ on $CD4^+$ T-cells as an attachment factor to increasing the efficiency of infection of target cells? Does ER-Env on virions activate $CD4^+$ T-cells through $\alpha_4\beta_7$ to promote proliferation and viral replication? Does ER-Env on infected cells interact with $\alpha_4\beta_7$ on bystander $CD4^+$ T-cells in order to stabilize a virological synapse? The answers to these questions will provide a better understanding of the role of non-fully-processed Env in HIV's interaction with $\alpha_4\beta_7$. They will also shine light on the broader subject of the role of $\alpha_4\beta_7$ in the spread of infection and transmission of HIV.

Immediately following the publication of the RV144 vaccine trial, our group was asked to determine if the anti-V2 response associated with reduced risk could inhibit $\alpha_4\beta_7$ binding. At that time, our group, and the field in general, lacked an assay capable of answering this question. The assays described in this thesis by our group and others suggest that it is now possible to revisit this question. Determining whether the response elicited by RV144 targets the $\alpha_4\beta_7$ epitope of V2 specifically and if it is capable of inhibiting the binding of $\alpha_4\beta_7$ to the V2 loop is a worthy of pursuit.

In conclusion, we describe throughout this thesis advances in our understanding of the regulation of HIV Env glycosylation and how the conformation in which Env is presented is critical for its interaction with integrin $\alpha_4\beta_7$. Our work has shown that the virus is capable of fine-tuning the glycan pattern of gp120 through natural variations in the SP

of Env that can shield neutralizing epitopes from building immune pressure in the host. We also show that an alternate, yet conserved, helical conformation of the V2 is the result of incompletely processed Env and is recognized by mAbs associated with protection (mAb CH58). This incompletely processed Env is present on the surface of infected cells and is also incorporated into virions. These helical-epitope-preferring mAbs are weakly neutralizing and inhibit the adhesion of $\alpha_4\beta_7$ to the V2 loop of gp120. These findings suggest a larger connection between HIV transmission, the interaction between gp120 and $\alpha_4\beta_7$, and the gut-tropic nature of HIV pathogenesis. Much remains to be determined about the complete impact of SP variation, the significance of CH58-reactive Env, and the role of $\alpha_4\beta_7^+CD4^+$ T-cells in HIV transmission and pathogenesis. These avenues will broaden our understanding of the early events of HIV transmission and the mechanisms by which HIV has adapted to overcome selective barriers. These approaches will aid in the development of improved HIV prevention and therapeutic strategies needed to combat the HIV epidemic.

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Chapter 5:
BIOGRAPHICAL SKETCH

5.1 Author Biography

Jason R. Yolitz was born in 1988 on Elmendorf, Air Force Base, in Anchorage, Alaska, USA, to a civil engineer father, Brian D. Yolitz, USAF Col. Ret., and a nurse mother, Dr. Annette H. Yolitz, DNP.

Jason received a Biotechnology High School Diploma from Osbourn Park High School in Manassas, Virginia in 2007 where he was first introduced to the molecular biology techniques and critical thinking skills necessary for biomedical research.

Jason completed his undergraduate work at Virginia Tech in Blacksburg, Virginia in 2011. While at Virginia Tech, Jason majored in Biological Sciences with a concentration in Biotechnology. Between his class schedule, he worked for three years as a Laboratory Technician under Dr. Arthur Buikema, Ph.D. assisting with the development of the Introductory Biology Laboratory course at Virginia Tech. Jason also worked as an Animal Care Technician maintaining colonies of mice, finches, frogs, fish and chameleons for research faculty in the Department of Biological Sciences.

In the fall of 2011, Jason joined the Translational Gerontology Branch of the National Institute of Aging (NIA) in Baltimore, Maryland as a Post-baccalaureate Research Fellow under Dr. Sige Zou, Ph.D. While at NIA, Jason worked on several projects studying the genetic and molecular mechanisms involved in lifespan and healthspan modulation using the model organism *Drosophila melanogaster*. His research contributions resulted in multiple publications in aging research journals and textbooks. His work on nutraceuticals was also covered by an article in The Wall Street Journal.

Jason began his graduate education in the fall of 2013 after joining the Johns Hopkins University – NIH Graduate Partnership Program. After spending a year taking

courses, conducting research rotations and teaching undergraduate students in Baltimore, Maryland at the JHU Homewood campus, Jason began his dissertation research as part of the Laboratory of Immunoregulation (NIAID) under Drs. James Arthos, Ph.D. and Anthony S. Fauci, MD. This dissertation is the result of his efforts to understand the role of post-translational processing of the HIV-1 Envelope protein on gp120 structure and function. It is his hope that this work will contribute to the global efforts to better understand HIV-1 transmission and pathogenesis as well as contribute to the effort to design a protective HIV-1 vaccine.

5.2 Author Curriculum Vitae

Jason Yolitz

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Education

September 2013 - December 2018 **Doctor of Philosophy**, Johns Hopkins University
Cell, Molecular, and Developmental Biology, and Biophysics

August 2007 - May 2011 **Bachelor of Science**, Virginia Tech
Biological Sciences

Research Experience

September 2013 - Present **Dissertation Research Fellow** under James Arthos, Ph.D. and Anthony S. Fauci, MD
Laboratory of Immunoregulation, NIAID, Bethesda, MD
Characterization of genetic influence on protein processing and glycosylation, and their impact on structural and functional characteristics of the HIV-1 Envelope protein

August 2011 - August 2013 **Postbaccalaureate Research Fellow** under Sige Zou, Ph.D.
Translational Gerontology Branch, NIA, Baltimore, MD
Studied molecular mechanisms involved in dietary interactions with genetic factors that modulate lifespan and healthspan in *Drosophila melanogaster*

Related Employment History

August 2008 - August 2011 **Laboratory Technician** under Arthur Buikema, Ph.D.
Department of Biological Sciences, Virginia Tech, Blacksburg, VA
- Aided in updating current experiments and development of new labs to be introduced into the *General Biology Lab* laboratory manual
- Prepared materials and solutions for all general biology laboratory course experiments

June 2009 - August 2009 **Animal Care Technician** under Bambi Jarrett
Department of Biological Sciences, Virginia Tech, Blacksburg, VA
- Provided general care for study animals including mice, finches, chameleons, fish and frogs

Additional Education

October 2016 **The Mass Spectrometry of Glycoproteins**
Complex Carbohydrate Research Center, *University of Georgia*
- Three-day hands-on course about the analysis of glycoproteins by mass spectrometry

August 2016 **Separation and Characterization of Glycoprotein and Glycolipid Oligosaccharides**
Complex Carbohydrate Research Center*, University of Georgia*
- Five-day hands-on course about current techniques of studying glycoproteins

August 2015 **Techniques in Glycobiology** taught by Natasha Zachara
Johns Hopkins University
-Two-week hands-on, intensive course on experimental techniques in studying glycosylation

June 2015 **Writing and Publishing a Scientific Paper** taught by Maggie Meitzler
Office of Intramural Training and Education, NIH
-Four-week workshop on scientific writing and the publishing process

January 2013 **Computing for Data Analysis** taught by Roger Peng and Jeff Leek
Coursera and *Johns Hopkins University*
- Four-week on-line course on exploring, analyzing and interacting with big data

Teaching Experience

Fall 2015 **Instructor**
Foundation for Advanced Education in the Sciences, Bethesda, MD
- Prepared and delivered lecture entitled 'Fluorescence and Flow Cytometry' to educationally diverse class of ~20 students

Spring 2015 **Instructor** and **Teaching Assistant**
Foundation for Advanced Education in the Sciences, Bethesda, MD
- Prepared and delivered lectures entitled 'Fluorescence and Flow Cytometry' and 'Nucleic Acid Biology' to educationally diverse class of ~20 students
- Organized and kept track of grade book, and graded exams throughout the semester

Spring 2014 **Graduate Student Teaching Assistant**
Department of Biology, Johns Hopkins University, Baltimore, MD
- Instructor for General Biology Lab II of ~20 undergraduate students
- Proctored and graded exams throughout the semester

Honors and Awards

2013 **Outstanding Poster Award**, NIH Postbac Poster Day
- For authoring a scientific poster that scored in the top 20% of all posters presented

2008,-10,-11 **Dean's List Recipient**, Virginia Tech
- For maintaining a 3.4 GPA or higher while attempting at least 12 credit hours per semester

Community Activities and Leadership

2018 **Participant**, *Biodefense Red Team Exercise,* JHU Center for Health Security, Baltimore, MD
- Red Team participant to identify potential biosecurity vulnerabilities in emerging technologies

2017-present **Member**, *International AIDS Society* (IAS)
- Association of international HIV researchers, civil society members and policymakers

2017 **Attendee**, *NIH FDA GlycoSciences Research Day*, Bethesda, MD
- Attended meeting on the role of carbohydrates in health and disease

2017 **Participant**, *US House Appropriations Committee on LHHS visit to NIH*, Bethesda, MD
- Student panel participant for the Congressional committee's visit to NIH

2016 **Lead Judge**, *NIH Postbac Poster Day Competition*, Bethesda, MD
- Organized group of four judges to critique and evaluate six postbac poster presentations

2016 **Attendee**, *International Conference on Emerging Infectious Diseases*, Bethesda, MD
- Attended AIDS panel of a meeting on transmission of emerging infections

2015 **Attendee**, *NIH FDA GlycoSciences Research Day*, Bethesda, MD
- Attended meeting on the role of carbohydrates in health and disease

2012 **NIA Representative**, *NIH Postbaccalaureate Committee*, Bethesda, MD
- Represented the NIA Baltimore campus postbacs on the NIH-wide Postbac Committee

2012 **Volunteer and Attendee**, *Metabolism, Diet and Disease Meeting*, Washington D.C.
- Volunteered at and attended meeting on biology of metabolism and disease

Funding and Scholarships

2017 **Intramural AIDS Research Fellowship**, NIH OAR, OITE and IR
- Full annual funding for outstanding scientific research and career development potential

2017 **Graduate Student Research Award**, NIH Graduate Student Research Symposium
- Travel funding for the highest rated poster in the Immunology/Virology/Bacteriology section

2016 **R21 NIH Exploratory/Developmental Research Grant Award**, AIDS MCB Study Section
- Awarded to collaborator *Chitra Upadhyay* (Mt. Sinai SOM) citing dissertation research data

2016 **Fellows Award of Research Excellence**, NIH
- Travel funding for a scientific abstract that scored in the top 25% of all abstracts submitted

Poster Presentations

Yolitz J, Wei D, Van Ryk D, Cicala C, Arthos J, Fauci AS. (January 2018). *Post-translational processing influences antibody reactivity against the gp120 v2 domain of infected CD4$^+$ T-cells.* **Keystone: Progress and Pathways Towards an Effective HIV Vaccine**, Banff, Canada.

Yolitz J, Wei D, Van Ryk D, Cicala C, Arthos J, Fauci AS. (July 2017). *Post-translational processing influences antibody reactivity against the gp120 v2 domain of infected CD4$^+$ T-cells.* **9th International AIDS Society (IAS) Conference on HIV Science**, Paris, France.

Yolitz J, Wei D, Van Ryk D, Cicala C, Arthos J, Fauci AS. (March 2017). *Post-translational processing influences antibody reactivity against the gp120 v2 domain of infected CD4$^+$ T-cells.* **13th Annual NIH Grad Student Research Symposium**, Bethesda, MD.

Yolitz J, Schwing C, Van Ryk D, Wei D, Nawaz F, Jelicic K, Fauci AS, Cicala C, Arthos, J. (February 2016). *The signal peptide of HIV Env influences glycosylation, antigenicity and DC-SIGN affinity of gp120.* **Conference on Retroviruses and Opportunistic Infections (CROI)**, Boston, MA

Yolitz J, Schwing C, Van Ryk D, Wei D, Nawaz F, Jelicic K, Fauci AS, Cicala C, Arthos, J. (January 2016). *The signal peptide of HIV Env influences glycosylation, antigenicity and DC-SIGN affinity of gp120.* **12th Annual NIH Grad Student Research Symposium**, Bethesda, MD.

Yolitz J, Sun X, Wheeler C, Laslo M, Alberico T, Sun Y, Song Q, Zou S. (June 2013) *Mitochondrial protein ATP synthase-d interacts with macronutrients to modulate lifespan in* Drosophila. **American Aging Association Meeting**, Baltimore, MD.

Yolitz J, Sun X, Wheeler C, Laslo M, Alberico T, Sun Y, Song Q, Zou S. (April 2013) *Mitochondrial protein ATP synthase-d interacts with macronutrients to modulate lifespan in* Drosophila. **NIH Postbac Poster Day**, Bethesda, MD.

Yolitz J, Sun X, Komatsu T, Lim J, Laslo M, Wang C, Poirier L, Alberico T, and Zou S. (June 2012) *SOD1 interacts with macronutrients to modulate lifespan in* Drosophila. **American Aging Association Meeting**, Fort Worth, TX.

Yolitz J, Sun X, Komatsu T, Lim J, Laslo M, Wang C, Poirier L, Alberico T, and Zou S. (April 2012) *SOD1 interacts with macronutrients to modulate lifespan in* Drosophila. **NIH Spring Research Festival**, Bethesda, MD.

Oral Presentations

Yolitz J, Wei D, Van Ryk D, Cicala C, Fauci AS, Arthos J. (October 2016) *The anti-gp120 mAb CH58 recognizes a not-fully-processed from of surface-expressed Env.* **HIV Research for Prevention (HIV R4P)**, Chicago, IL.

Publications

Wibmer CK, Richardson SI, **Yolitz J**, Cicala C, Arthos J, Moore PL, Morris L. (2018). *A common helical conformation of V1V2 exposes the $\alpha_4\beta_7$ binding site on intact HIV-1 virions.* **Nat Commun**. 9(1): p. 4489.

Lertjuthaporn S, Cicala C, Van Ryk D, Liu M, **Yolitz J**, Wei D, Nawaz F, Doyle A, Horowitch B, Park C, Lu S, Lou Y, Wang S, Pan R, Jiang X, Villinger F, Byrareddy SN, Santangelo PJ, Morris L, Wibmer CK, Biris K, Mason RD, Gorman J, Hiatt J, Martinelli E, Roederer M, Fujikawa D, Gorini G, Franchini G, Arakelyan A, Pattanapanyasat K, Kong X-P, Ansari AA, Fauci AS , Arthos J. (2018). *Select gp120 V2 domain specific antibodies derived from HIV and SIV infection and vaccination inhibit gp120 binding to $\alpha_4\beta_7$.* **PLOS Pathog.** 14(8): p. e1007278

Yolitz J, Schwing C, Chang J, Van Ryk D, Wei D, Nawaz F, Jelicic K, Cicala C, Arthos J, Fauci AS. (2018). *Signal peptide of HIV envelope protein impacts glycosylation and antigenicity of gp120.* **PNAS.** 115 (10): 2443-2448.

Sun X, Wheeler C, **Yolitz J**, Laslo M, Alberico T, Sun Y, Song Q, Zou S. (2014). *A mitochondrial ATP synthase subunit interacts with TOR signaling to modulate protein homeostasis and lifespan in Drosophila*. **Cell Reports**. 8(6):1781-1792.

Sun Y*, **Yolitz J***, Alberico T, Sun X, Zou S. (2014). *Lifespan extension by cranberry supplementation partially requires SOD2 and is life stage independent*. **Exp. Gerontol**. 50: 57-63. (*Authors contributed equally to this work)

Sun Y, **Yolitz J**, Wang C, Spangler E, Zhan M, Zou S. (2013). 'Aging Studies in *Drosophila melanogaster*.' In ***Biological Aging***, pp. 77-93. Humana Press.

Wang C, **Yolitz J**, Alberico T, Laslo M, Sun Y, Wheeler T, Sun X, Zou S. (2013). *Cranberry interacts with dietary macronutrients to promote healthy aging*. **J Gerontol A Biol Sci Med Sci**. 69(8): 945-954.

Sun X, Komatsu T, Lim J, Laslo M, **Yolitz J**, Wang C, Poirier L, Alberico T, Zou S. (2012). *Nutrient-dependent requirement for SOD1 in lifespan extension by protein restriction in Drosophila melanogaster*. **Aging Cell**, 11: 783-793.